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Arylamidase activity of soils

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Arylamidase activity of soils

by

Verónica Acosta-Martínez

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Soil Science (Soil Microbiology and Biochemistry)

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Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

Verónica Acosta-Martínez

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College

DEDICATION

To my family, especially my son Sergio José

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INTRODUCTION

Soil, as a living system contains many enzymes. Most soil enzymes are primarily of microbial origin, but may also originate from animal and plant residues (Kiss et al., 1975; Burns, 1982). Enzymes are present in soils inside (intracellular) living cells, in the periplasmic space and outer cell surfaces stabilized on the clay-organic matter complexes (Burns, 1982). Soil enzymes play a key role in the decomposition of various organic compounds, and in the decomposition and the formation of the soil organic matter. Thus, enzymes are important components involved in the dynamics of the cycling of the nutrients C, N, P, and S in soils.

Among the most important nutrients in soils, N is the most needed by most agricultural crops but only trace quantities are available in the mineral form. The organic form is a major component of soil organic matter and may account for greater than 95% of the total N in most surface soils. About half of this organic N has not yet been identified. It has been estimated that about 20-40 % of the total N in soils is present in the form of amino acids, but only a small portion of the amino acids is present in a "free" state and the major portion is bound to soil organic matter (Bremner, 1951). Several researchers (Grov, 1963; Grov and Alvsaker, 1963; Ivarson and Sowden, 1966, 1970; Paul and Schmidt, 1960; Stevenson, 1982, 1985; Monreal and McGill, 1985) have studied the portion of "free" amino acids in soils because it is an available energy source for

soil microorganisms (Ivarson and Sowden, 1966) and an available N source for plant growth (Broadbent, 1984). The amino acids bound to the soil organic matter are most likely in the form of proteins or associated to the clay-organic matter complexes as peptides, amides or arylamides. These amino acids are released from the soil organic matter by the activities of enzymes such as arylamidase, and later the "free" amino acids are hydrolyzed by specific enzymes, producing NH_4^+, which, in turn, is nitrified for plant uptake.

Among the amidohydrolases, L-aspartase, L-asparaginase, and L-glutaminase are enzymes acting on "free" amino acids of soils. The amidohydrolases are very specific in that they act on the C-N bonds other than peptide bonds in linear amides releasing NH_4^+. Many researchers have reported about the activities of such amidohydrolases and the factors that affect their activities (Frankenberger and Tabatabai, 1991c,d; Tabatabai, 1994; Senwo and Tabatabai, 1996,1999).

The enzyme amino acid arylamidase [α-aminoacyl-peptide hydrolase (microsomal) EC 3.4.11.2] catalyzes the hydrolysis of an N-terminal amino acid from peptides, amides, or arylamides. This enzyme has been detected in the tissues and body fluids of all animals (Hiwada et al., 1980), plants, and microorganisms (Appel, 1974). Arylamidase may play a key role in the initial reactions of the mineralization of amino acids in soils but no information is available about this enzyme in soils. Studies to understand the role of arylamidase in soil N cycling and the factors (i.e., soil properties, trace elements,

liming, tillage and crop residues management practices) that affect the activity of this enzyme will aid in decision making important for the fertility, productivity and sustainability of soils.

The objectives of this work were: (1) to develop a method to assay the activity of arylamidase in soils, (2) to assess the factors affecting arylamidase activity in soils and its relationship to the amidohydrolases, (3) to assess the effect of trace elements on the activity of this enzyme in soils, (4) to determine the effect of toluene on the activity of arylamidase, (5) to study the substrate specificity of arylamidase, (6) to evaluate the effect of liming on the activities of 14 different enzymes, including arylamidase, and (7) to evaluate the influence of tillage and residue management on the activity of arylamidase.

The results obtained are presented in six parts. The results obtained under objective (1) are presented in Part I, those under objective (2 and 3) are presented in Part II, those under objective (4) are presented in Part III, those under objective (5) are presented in Part IV, those under objective (6) are presented in Part V, and those under objective (7) are presented in Part VI. Preceding Part I is a literature review and after Part VI is a summary and conclusions.

LITERATURE REVIEW

Soil Enzymes

Soil is a living system where all biochemical activities proceed through enzymatic processes. About a century ago, Woods reported about oxidizing enzymes, especially peroxidases, in soils (cited by Skujins, 1978). Since then many enzymes have been detected in soils, and now soils may looked upon as biological entities, that is, living tissues (Quastel, 1946). The history of soil enzyme research is summarized by Skujins (1967; 1976), Ladd (1985), and Tabatabai (1994). The most progress has been done since the 1960's when serious effort was made to develop methods for assay of enzyme activities in soils. Since then, the annual number of papers published in this field increased exponentially, and now methods are available for assaying numerous enzyme activities in soils, especially the enzymes involved in C, N, S, and P cycling in soils (Tabatabai, 1994).

Enzymes are proteins that act as catalysts without undergoing permanent alteration and cause chemical reactions to proceed at faster rates. Enzymes are specific activators because they combine with their substrates in stereospecific fashion that decreases the stability of certain susceptible bonds (i.e., changes in electronic configuration). Enzymes are present in soils in living and dead plant and animal material cells. It is generally assumed, however, that soil enzymes are largely derived from microbial origin. Some of the pioneers in the study of

soil enzymes have provided valuable information about the different pools of enzymes in soils. In a review of the literature, Skujins (1976) stated that the total soil enzyme activity depends on the level of extracellular enzymes, active enzymes from dead cells and the activity from living or proliferating cells. Most of the enzymes added to soils by decaying microbial tissues and plant and animal residues are likely degraded by soil proteases, and what remains is incorporated with the humus. Kiss et al. (1975) concluded that the enzymes accumulated in soils are present as free enzymes (exoenzymes released from cells), endoenzymes released from disintegrated cells, enzymes bound to cell constituents (such as disintegrated cells, in cell fragments in viable but non-proliferating cells). Soil, therefore, can be viewed as a system of humus and minerals containing both immobilized enzymes and occluded microbial cells (McLaren, 1975). The total activity of an enzyme in soils is comprised of activities associated with different soil constituents and 10 distinct categories of soil enzymes may exist (Burns, 1982): (i) enzymes which function within the cytoplasm of proliferating microbial, animal and plant cells, (ii) enzymes restricted to the periplasmic space of the proliferating Gram-negative bacteria, (iii) enzyme attached to the outer surface of the viable cell yet whose active sites extend into the ambient medium, (iv) enzymes which are secreted by living cells during normal cell growth and division and are found in the aqueous phase of the soil, (v) enzymes within non-proliferating cells such as fungal spores, protozoa cysts, plant seeds and bacteria endospores, (vi) enzymes attached to an

entire dead cell and cell debris, (vii) enzymes which leak from extant cells or are released from lysed cells, whose original functional location was on or within the cell and which may survive for a short period in the aqueous phase of the soil, (viii) enzymes which are associated temporarily in soluble or insoluble enzyme-substrate complexes, (ix) enzymes which become absorbed to clay minerals either on the external surfaces or within the lattices of 2:1 layer silicates, (x) enzymes which become associated with humic colloid due to absorption, entrapment, or co-polymerization during humic matter genesis.

Systematic studies have been done to optimize the assay conditions needed to measure the activities of more than 15 different enzymes present in soils (Casida, 1964; Tabatabai, 1994; Senwo and Tabatabai, 1996). The factors tested include buffer pH, substrate concentration, time of incubation, temperature of incubation, pre-heating temperature, and amount of soil. For example, such factors have been studied for the development of the assay methods of the activities of soil amidohydrolases as they affect the release of NH_4^+ from the substrates (Ladd and Jackson, 1982; Tabatabai, 1994; Senwo and Tabatabai, 1996).

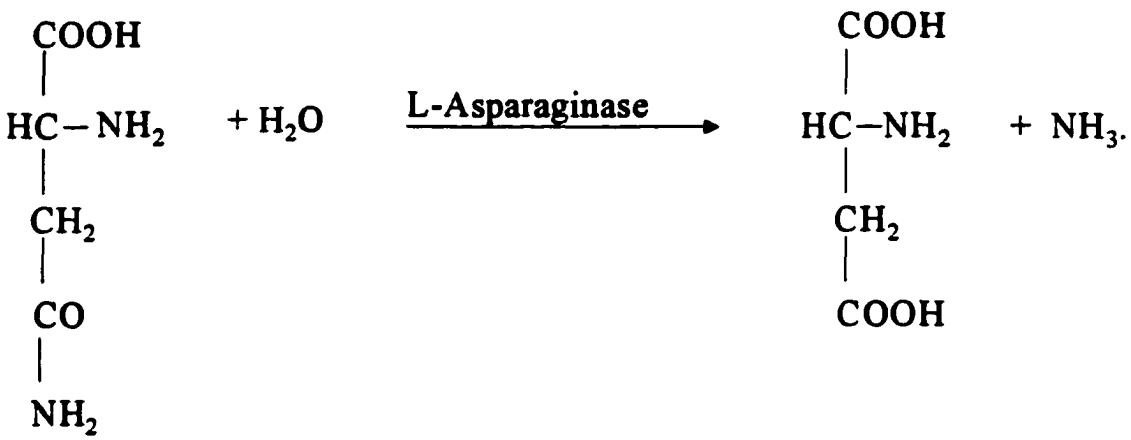
Soil enzyme assays can complement chemical soil tests and thus, can be a practical tool for soil sustainability because they may provide an index of soil fertility. The numbers obtained from each enzyme assay may be seen as an indication of the nutrient status of soils. Many have reported the decreases in phosphatase activity due to increases in the P solution concentrations by

applications of P fertilizers (Nannipieri et al., 1978; Speir and Ross, 1978). Dick et al. (1988b) reported decreases in the activities of amidase and urease due to the increasing rates of ammonia based N fertilizer to plots established since 1931. Conversely, activities of other soil enzymes such as arylsulfatase and β - glucosidase did not correlate with the N fertilizer applications. These results demonstrated there is a feedback mechanism that suppresses the production of some enzymes whose reaction products are added by the inorganic fertilizer. Dick (1994) suggested carefully selecting the enzyme that may be the best indicator of changes in the soil nutrient concentration by the fertilization practice.

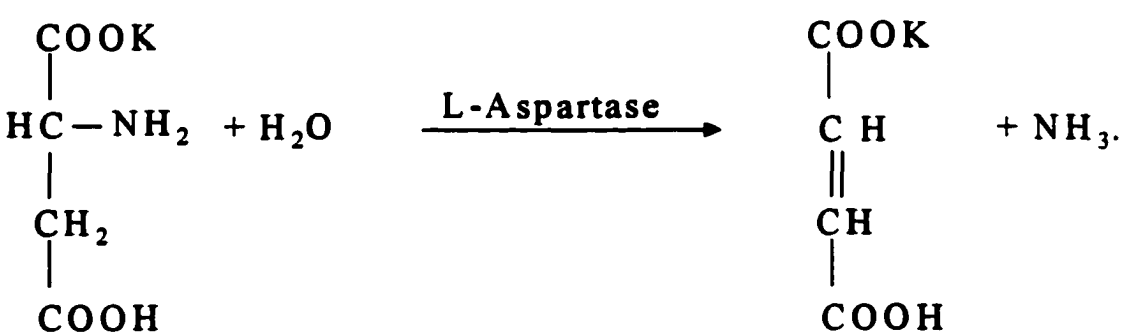
Amidohydrolases

The chemical nature of N in soils is such that a large proportion (15-25%) of organic N is often released as NH_4^+ by 6 *M* HCl hydrolysis. Amidohydrolases such as L-asparaginase, L-aspartase, L-glutaminase, amidase and urease are very important in the soil N cycle. With the exception of urease, all these soil enzymes are considered to play an important role in N mineralization in soils. Amidohydrolases cleave C-N bonds other than peptide bonds in linear amides and release NH_3 (Ladd and Jackson, 1982; Tabatabai, 1994). The amide hydrolysis is a nucleophilic displacement reaction in which the displaced N atom leaves as an NH_4^+ group (Ladd and Jackson, 1982). Similar to all other enzymes, the amidohydrolases are very specific to their substrates. L-

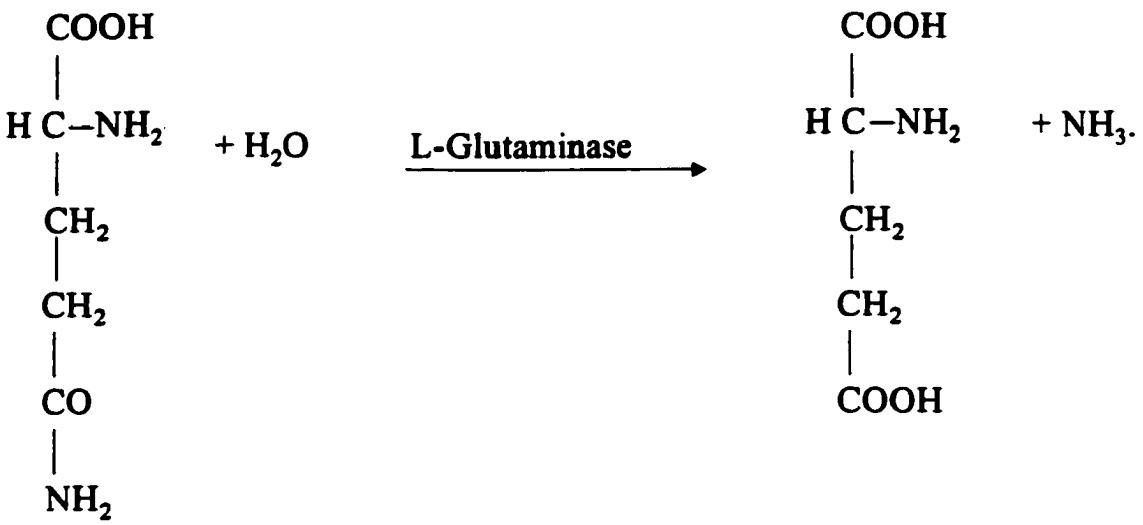
asparaginase (L-asparaginase amidohydrolase, EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine producing aspartic acid and NH_3. The reaction is as follows:



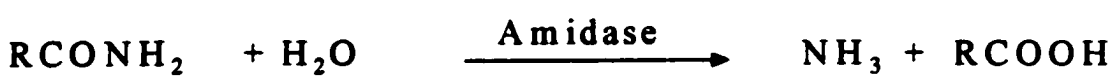
L-Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the hydrolysis of aspartate, produced in the reaction catalyzed by L-asparaginase, and produces fumarate and NH_3. The reaction is as follows by using its K form:



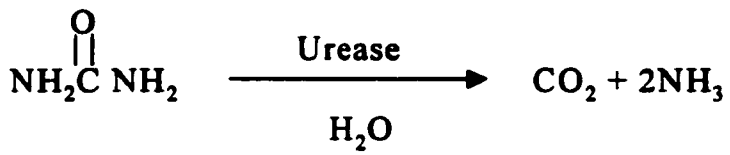
The reaction catalyzed by L-glutaminase (EC 3.5.1.2) involves the hydrolysis of L-glutamine to L-glutamic acid and NH_3 as follows:



Amidase (acylamide amidohyrolase, EC 3.5.1.4) catalyzes the hydrolysis of amides and produces NH_3 and the corresponding carboxylic acid according to the following reaction:



Urease (urea amidohydrolase, EC 3.5.1.5), although not involved in N mineralization in soils, catalyzes the hydrolysis of urea, added to soils as a fertilizer, and produces CO_2 and NH_3. The reaction of this enzyme is as the following:

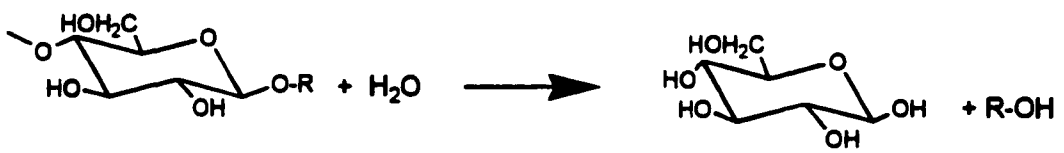


Glycosidases

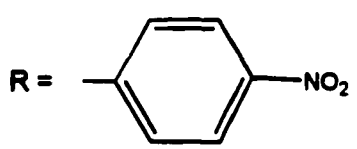
The glycosidases are the group of enzymes that act on the glycosyl compounds including the glycoside hydrolases (EC 3.2.1): α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), α-galactosidase (EC 3.2.1.22), and β-galactosidase (EC 3.2.1.23). The glycosidases are widely distributed in nature (Bahl and Agrawal, 1972; Dey and Pridham, 1972; Wallenfels and Weil, 1975) including soils (Skujins, 1967, 1976) where they play a key role in the breakdown of low molecular-weight carbohydrates, producing sugars, the main energy source to soil microorganisms.

This group of enzymes has been named according to the type of bond that they hydrolyze. For example, α-glucosidase, also named maltase, catalyzes the hydrolysis of α-D-glucopyranosides (maltose). β-glucosidase, also named gentibiase or cellobiase, catalyzes the hydrolysis of β-D-glucopyranosides

(cellobiose). The enzyme α-galactosidase, also named melibiase, catalyzes the hydrolysis of α-D-galactopyranosides, and β-galactosidase, also named lactase, catalyzes the hydrolysis of β-D-galactopyranosides. Among all the glycosidases, β-glucosidase is the most predominant enzyme in soil (Eivazi and Tabatabai, 1988, 1990). The general reaction of the glucosidases is shown below using as an example the reaction catalyzed by β-glucosidase:



p-nitrophenol-β-D- glucoside β-D-glucopyranose *p*-nitrophenol



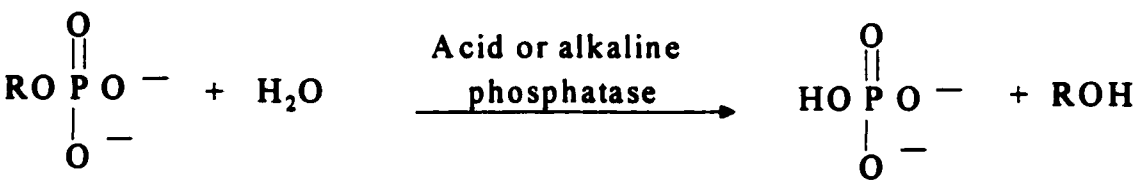
Phosphatases and Arylsulfatase

The elements phosphorus (P) and sulfur (S) are essential for plant growth and metabolism. Both elements have received a great deal of study because their transformations in the soil system are not completely understood, but it is generally accepted that they are taken up by plant roots in their inorganic forms.

Because P and S are present in organic forms in most surface soils, the mineralization of these organic fractions is very critical for plant growth and development. Phosphatases and arysulfatase play a major role in the mineralization processes of soil organic P and S, respectively.

The term *phosphatases* have been used to describe a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of H_3PO_4 (Schmidt and Laskowski, 1961). According to the commission on enzymes of the International Union of Biochemistry, there are five groups of phosphatases: phosphoric monoester hydrolases (EC 3.1.3), phosphoric diester hydrolases (EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5), enzymes acting on phosphoryl-containing anhydrides (EC 3.6.1), and enzymes acting on P-N bonds (EC 3.9), such as the phosphoamidase (EC 3.9.1.1). The most common groups of phosphatases studied in soils are the phosphomonesterases --acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1)--, and phosphodiesterase (EC 3.1.4) (Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977; Browman and Tabatabai, 1978). The enzymes are classified acid and alkaline phosphatases because they show optimum activities in acid and alkaline ranges, respectively. Because of the importance of these enzymes in soil organic P mineralization and plant nutrition, considerable literature has accumulated on the phosphomonoesterases in soils (Eivazi and Tabatabai, 1977; Speir and Ross, 1978; Tabatabai, 1994). Eivazi and Tabatabai (1977) assayed the phosphatase activity at different buffer pH values and demonstrated that acid phosphatase

and alkaline phosphatase were predominant in acid and calcareous soils, respectively. They concluded that the production, stability, and distribution of acid and alkaline phosphatase activities are correlated to soil pH. These findings are very important in predicting the type of soil enzymes that are induced under given management practices (i.e., lime and fertilization) or environmental conditions that tend to change the soil pH. Studies have reported that higher plants are devoid of alkaline phosphatase activity (Dick et al., 1983; Juma and Tabatabai, 1988a,b,c), and thus, the alkaline phosphatase activity in soils seems derived totally from microorganisms. Following is the reaction involved in hydrolysis of phosphomonoesters by acid and alkaline phosphatases at their optimal buffer pH of 6.5 or 11 in soils, respectively.



The reaction catalyzed by phosphodiesterase (orthophosphoricdiester phosphohydrolase, EC 3.1.4.1) is as follows; where the R_1 and R_2 represent either alcohol or phenol groups or nucleosides (Privat de Garilhe, 1967):

Several types of sulfatases occur in soils, and as is the case of glycosidases or phosphatases, they have been classified according to the type of bond (organic sulfate esters) they hydrolyze. The groups of enzymes included in this group are: arylsulfatases, alkylsulfatases, steroid sulfatases, glucosulfatases, chondrosulfatases, and myrosulfatases (Fromageot, 1950; Roy, 1960). Among the sulfatases, the activity of arylsulfatase was first detected in soils by Tabatabai and Bremner (1970), and a procedure was developed for its assay. This enzyme is believed to be partly responsible for the S cycling in soils. The suggested role of arylsulfatase in S mineralization is largely derived from studies showing that between 40 and 70 % (avg. 50%) of the total S in surface soils of temperature regions is reduced to H_2S by HI and is converted to inorganic SO_4^{2-} with hot alkali (Freney, 1961; Tabatabai and Bremner, 1972; Neptune et al., 1975). Arylsulfatase (arylsulfate sulfohydrolase, EC 3.1.6.1) catalyzes the hydrolysis of an arylsulfate anion by fission of the O-S bond (Spencer, 1958). The reaction is as follows:

$$ROSO_3^- + H_2O \xrightarrow{\text{Arylsulfatase}} ROH + H^+ + SO_4^{2-}.$$

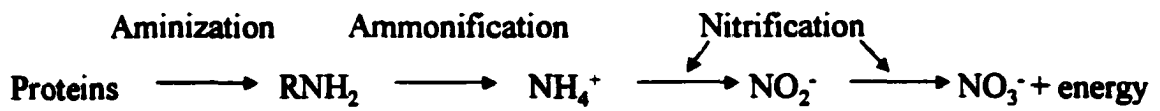
Nitrogen Mineralization

Nitrogen (N) is an essential nutrient for plant growth and development and the most deficient in soils. More than 95% of the total in most soils is

organic in nature. Organic N in soils is not available to plants; it has to be mineralized to inorganic forms for plant uptake.

Studies on the distribution of the major organic N compounds in soils, obtained from different climatic and geological conditions, showed that despite the variations in total N content among the soils, the proportions of total N that could be hydrolyzed by hot HCl (6M) were quite similar (84.2 % to 88.9 %) (Sowden et al., 1977). In general, it has been shown that 20 to 50 % of the total N in soils is in the form of bound amino acids, 5-10 % is in the form of combined hexosamines (combined aminosugars), < 1% purines and pyrimidine bases, and the rest of organic N compounds in soils remain unidentified.

The inorganic N intermediates produced from the reactions involved in soil N mineralization are: NO_2^-, NO_3^-, exchangeable NH_4^+, non-exchangeable (mineral fixed) NH_4^+, dinitrogen gas (N_2), and nitrous oxide (N_2O). Soil microorganisms play the major role in such biological N transformations. An example of the succession of reactions involved in N mineralization under aerobic conditions is as follows:



Aminization and ammonification processes are dependant on heterotrophic microorganisms and enzymes produced, and nitrification of NH_4^+

to NO_2^- and NO_3^- is carried out by the obligate autotrophic aerobic soil bacteria including the species *Nitrosomas* and *Nitrobacter*, respectively.

Organic materials (e.g., plant residues, biotechnology by-products, animal manures) added to soils are also transformed by soil microorganisms to inorganic N forms through the process of mineralization. Several factors may influence N mineralization in soils as well as N immobilization (or tie-up); the process that governs the incorporation of N into soil organic matter by soil microorganisms. The activity of the microorganisms responsible for N mineralization (as with other microorganisms), and therefore, the rates and products of N mineralization are affected by environmental conditions such as moisture, soil pH, and temperature. Moisture is the most critical factor that affects the soil N mineralization and immobilization processes. Under aerobic conditions, the NO_3^- form is the most predominant end-product of N mineralization, whereas under anaerobic conditions induced in waterlogged soils, NH_4^+ is the predominant end-product. Moisture also affects the movement and, thus, the availability of NO_3^- in soils. Although several researchers have reported different results about the effects of moisture on the N mineralization rates under aerobic conditions, the general trend is an adverse effect on both extremes of soil moisture.

A review on the effect of soil acidity on soil biochemical processes concluded that nitrification, as well as other biochemical processes (e.g., denitrification, glucose degradation), are greatly affected by the changes on soil

pH (Tabatabai, 1985). A study by Fu et al. (1987) showed that a lag period is developed before crop residues added to soil are mineralized. This lag period was decreased as the soil pH was increased from 4 to 8.

Nitrogen mineralization rates are also affected by the changes in temperature. In frozen soils, the N mineralization becomes slower because of the restricted microbial activity. In addition, experimental evidence suggests that N mineralization proceeds differently under fluctuating temperature conditions than it does at constant temperature (Das et al., 1995).

The mineralization of N in soils is also affected by management practices, such as cropping systems and the applications of lime and fertilizers. A study by Deng and Tabatabai (2000) reported that soybean growth depleted soil organic matter and reduced net N mineralization, while the inclusion of meadow in the cropping systems enriched organic N which resulted in increased N mineralization. Between the rotations used in the study the meadow-based multiple crop systems were always advantageous over monocropping systems with respect to increase N mineralization rates, and thus supplying N for plant nutrition. Jenkinson and Ladd (1981) found that the greater microbial biomass and particulate organic matter accumulated in reduced tillage systems result in larger pools of easily mineralizable N. Compared with continuous moldboard plow, conservation tillage practices result in higher microbial biomass (Saffigna et al., 1989; Carter, 1992; Angers et al., 1993a), and thus, in mineralization rates (El-Haris et al., 1983; Campbell et al., 1991) due to the accumulation of plant

residues at or near the soil surface, which provides larger total amounts of organic C (Logan et al., 1991) and of organic components, such as soluble and acid-hydrolysable carbohydrates (Angers et al., 1993). Mineralization (i.e., rate of N release) of the crop residues left in soils not only depends on the environmental factors but also on the type of crop, the part and amounts of plant residues that are returned to the soil, and its contents of N, S, soluble C, lignin, and carbohydrates (Janzen and Kucey, 1988). The relative amounts of organic C to organic N (C:N ratio) of the crop residue returned to soil is a key factor influencing not only the mineralization process but also the immobilization process. Most reviews on C:N ratios indicate that plant residues, with wider C:N ratios (C:N > 30), incorporated into soils favor N immobilization and those with narrower ratios (C:N < 20) favor N mineralization (N release) early in the decomposition process.

Acidification leads to significant changes in microbial dynamics and consequently in nitrification rates. Studies by Karmarkar and Tabatabai (1991) showed that certain organic acids either inhibit or activate nitrification in soils. The study reported that aliphatic acids and aromatic acids showed to decrease the accumulation of NO_2^--N. Thus, liming soils and increasing soil pH may induce significant favorable changes to the soil environment of the microorganisms and the associated enzyme systems, and, thus, it is reflected on the N transformations. A study showed that liming increased the *in situ* mineralization of soil N over a 3-year period (Nyborg and Hoyt, 1978). Simard

(1994) reported that lime had a temporary effect on N mineralization, which disappeared after the second year in a Luvisolic soil under tillage treatments.

Different methods have been proposed to measure N mineralization in soils. Stanford and Smith (1972) developed a method that involves leaching of soil columns after incubation under aerobic conditions and determination of the inorganic N produced with steam distillation. Waring and Bremner (1964) proposed a method that involves estimation, by steam distillation, of the NH_4^+ produced under anaerobic conditions; under waterloogged conditions. In addition to the methods proposed for estimation of N mineralization in soils, assay of the activities of the enzymes involved in the N cycle, such as the amidohydrolases, is an important means of assessing the potential of soils to release inorganic N. Measurement of the activities of L-asparaginase, L-aspartase, L-glutaminase, and amidase, and studies of the factors that affect this group of enzymes promises to provide a better understanding of the biochemical reactions involved in N mineralization in soils.

Arylamidase

Arylamidase (EC 3.4.11.2] is the enzyme that catalyzes the hydrolysis of an N-terminal amino acid from peptides, amides or arylamides. This enzyme is widely distributed in the tissues and body fluids of all animals (Hiwada et al., 1980), plants, and microorganisms (Appel, 1974). The chemical nature of N in soils is such that a large proportion (15-25%) of organic N is often released as

NH_4^+ by 6 *M* HCl hydrolysis. The information available suggests that a portion of the released NH_4^+ is derived from amino acid residues in linear amides and arylamides of soil organic matter (Sowden, 1958). The activity of this enzyme in soils deserves investigation because present knowledge indicates that a variety of arylamides are present in soils (Stevenson, 1994). Arylamidase may play an important role as an initial limiting step in mineralization of organic N in soils.

In 1954 Gomori developed a precise colorimetric and histochemical method to study aminopeptidase in organ tissue based on the formation of azo dyes from the naphthylamine moiety liberated by enzymatic hydrolysis of glycyl- or alanyl- β-naphthylamide. The quantitative measurement of the enzyme amino acid arylamidase [α-aminoacyl-peptide hydrolase (microsomal) EC 3.4.11.2] was introduced by Goldbarg and Rutenburg in 1958 as an aid to the diagnosis of the cancer of the pancreas.

In the 50's arylamidase was confused with leucine aminopeptidase (EC 3.4.11.1). Goldbarg and Rutenburg (1958) used L-leucine β-naphthylamide as the substrate but still called the enzyme leucine aminopeptidase. Because the specific role of these two different enzymes was not clear in the 50's, Patterson et al. (1963) performed experiments to distinguish between leucine aminopeptidase and the enzymes that hydrolyze L-leucyl-β-naphthylamide. The experiments involved the following: (i) localization of the enzymes that hydrolyze L-leucineamide and L-leucine β-naphthylamide in fractions of ascites tumor cells, (ii) studies of the stability of the enzymes in soluble fractions by various

treatments, and (iii) separation of these enzymes by column chromatograph, and gel filtration to characterize the purified enzymes. To assay the activity of the enzymes that hydrolyze L-leucine β-naphthylamide they used the method described by Goldbarg and Rutenburg (1958), which involve diazotization of the product of the reaction (β-naphthylamine) with sodium nitrite, and conversion to an azo compound (blue azo dye) by reaction with N-(1-Naphthyl) ethylenediamine dihydrochloride. Patterson et al. (1963) found that the enzyme that hydrolyzes leucine aminopeptide is different from the enzyme that hydrolyzes the amino acid attached to β-naphthylamide. This conclusion was derived from experiments showing that leucine aminopeptidase hydrolyzes unsubstituted amides and peptides that posses a free α-amino group of the L-configuration. They also reported that the rate of hydrolysis with leucine aminopeptidase depends on the size and polarity of the R group (amino acid) on the carbon atom containing the free α-amino group, and that the nature of R' group on the peptide or amide nitrogen also influences the rate of hydrolysis. With respect to the substrate L-leucine β-naphthylamide, they reported that the R' group consists of the β-naphthyl ring, as the nitrogen bonded to the amino acid is connected directly to a ring carbon and that it seems to have more influence than the R group (amino acid) in conferring specificity on the enzymes that hydrolyze it. Nachlas and coworkers (1962) suggested the name "aryl aminopeptidase" be used for what is now known as arylamidase. Patterson and coworkers (1963) concluded that since it is the arylamides of amino acids that

are substrates for these enzymes, it would be more accurate to use the name "amino acid naphthylamidases" or more generally "arylamidases". Since then this name has been used for the enzyme hydrolyzing neutral amino acid β-naphthylamides.

The study by Nachlas et al. (1962), comparing the activity of arylamidase towards seven substrates consisting of different amino acid amides of β-naphthylamine, showed that it is unrealistic to characterize the enzyme by the amino acid used. Other researchers found that the activity of this enzyme was not significantly different when the substrates L-alanine or L-leucine arylamide were used for samples of the urine or intestine (Marks et al., 1968; Hiwada et al., 1977).

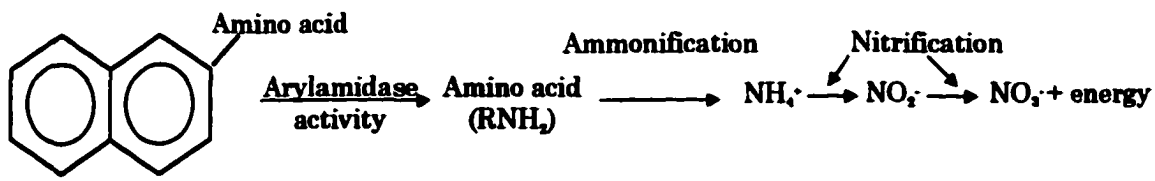
Studies conducted in the 70's showed that neutral amino arylamidase (EC 3.4.11.2) is distinct from the enzyme, leucine aminopeptidase (EC 3.4.11.1) which hydrolyzes L-leucine amide or L-leucylglycine (Patterson et al., 1963; Marks et al., 1968; Hiwada et al., 1977). Since then the term neutral arylamidase (EC 3.4.11.2) is used for the group of enzymes which is capable of readily splitting the neutral amino acid β-naphthylamides or the neutral amino acid p-nitroanilides (Patterson et al., 1963; Smith et al., 1965; Hiwada et al., 1977).

Studies with pure preparations of arylamidase from living systems, including human origin, have provided knowledge about the biochemical properties of this enzyme. Despite the different sources (e.g., human urine or

intestines and rat brain), the enzyme has shown similarities in terms of molecular weight, inhibitors of its activity, and the K_m values. By using enzyme purified from the small intestine, lung, kidney and other organs of humans, Hiwada et al. (1977) reported a molecular weight of 2.4×10^5 *Da*, an optimum activity of this enzyme within a pH range of 6.2 to 7.8, and the K_m value of 8.7×10^{-5} M. Marks et al., (1968) exposed the enzyme to different metals in the form of SO_4^{2-} or Cl^- at a concentration of 0.1 m*M*, and found that Co^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+} and La^{2+} activated arylamidase activity, whereas Cd^{2+}, Cu^{2+}, and Hg^{2+} almost completely inhibited the enzyme activity.

In soils, the presence of arylamidase has not been explored, even though recent information indicates that a variety of arylamides are present in soils (Stevenson, 1994). The study of arylamidase is very important to solve a puzzle on the chain of reactions that occur in the N cycling in soils. Arylamidase may play an important role as an initial limiting step in the hydrolysis the amino acids in soils (the aminization reaction).

The following figure shows the fate of amino acids under aerobic conditions after they are released from the soil organic matter by the activity of arylamidase:



Soil Quality

Soil quality has been defined as the capacity of a soil to function within its ecosystem boundaries to sustain biological activity and productivity, maintain environmental quality, promote plant and animal health, and habitation (Doran and Parkin, 1994; Gregorich et al., 1994). The quality of a soil impact farm productivity, land use capability, agronomic sustainability and the environmental buffering capacity. An assessment of soil quality that includes measurement of some biological, chemical, and physical properties can provide valuable information for determining the status of such soil parameters, their influence on the ecosystem boundaries, and the sustainability of soil management (Doran et al., 1994). The physical, chemical, and biological parameters of the soil are correlated with each other, and therefore any change of one of these soil parameters will affect the other. For example, changes in physical and chemical properties of soil affect the organic matter and moisture contents, which, in turn, affect microbial dynamics (Turco et al., 1994). The increase in soil organic matter is linked to a number of desirable soil physical, chemical, and biological properties that are associated with soil productivity (Stevenson, 1982). The presence of organic matter will control the soil's capacity to supply nutrients to plants, and modulate chemical and water availability (Nelson and Sommers, 1982). Organic matter improves soil structure and aggregation and is related to microbiological parameters such as the size of the

microbial biomass (Goyal et al., 1992) and soil enzyme activities (Frankenberger and Dick, 1983).

Management practices such as the application of organic amendments, fertilizer and/or lime to soils will affect the interaction of key soil physical, chemical, and biological parameters. While each parameter is a valuable indicator of soil quality, their response to changes is different. In agricultural ecosystems that have maintained cropping and management practices for a long period of time (*ca.*100 years), valuable information related to the quality of soils can be obtained from their physical and chemical properties. However, in those systems where changes are recent, the soil biological and biochemical parameters can be more sensitive indicators and provide earlier indications of change in soil quality, long before changes in total soil organic C and N can be detected by chemical analysis.

Studies have shown the soil biological and biochemical processes are an integral part of soil quality (Dick, 1992; Turco et al., 1994). Nannipieri (1983) found a faster response to changes in soil organic matter, indicated by increases in microbial biomass, than with the actual changes in the soil organic C content. Powlson et al. (1987) demonstrated that small changes in total soil C and N are difficult to detect against the large background levels of C and N in soils.

Soil microorganisms and their associated processes are potentially one of the most sensitive and earliest biological indicators of soil quality. The microbial biomass C only constitutes 1-4% of total soil organic C (Anderson and Domsch,

1989) but it plays a key role in the decomposition of organic materials in soils and it is also the major source of soil enzymes. Soil enzymes activities are considered indicative of specific biochemical reactions of the entire microbial community in soils, because they are involved in the transformations of the main soil nutrients C, N, S, and P (Frankenberger and Dick, 1983; Nannipieri et al., 1990).

Recently, soil quality investigations, including the measurement of the soil enzyme activities, has shown to provide information needed for management and regulatory decision making in soil systems, including agricultural, forestry or turfgrass, and any of those systems in disturbed or contaminated conditions. The activities of the soil enzymes have shown to be sensitive to soil management practices, including cropping systems (Khan, 1970; Dick, 1984; Bolton et al., 1985; Klose et al., 1999), and tillage treatments (Dick, 1984; Gupta and Germida, 1988; Deng and Tabatabai, 1996a,b; 1997) and to the applications of organic amendments (Yaroshevich, 1966; Khan, 1970; Verstraete and Voets, 1977; Dick et al., 1988; Martens et. al., 1992).

Liming Effect on Soil Quality

Soil acidification occurs due to several factors, including: the accumulation of H_2CO_3 formed from high concentrations of CO_2 in the soil atmosphere produced by plant roots and microbial respiration, the mineralization of organic N and S, fixation of N by leguminous plants, NH_4^+- forming fertilizers,

nitrification of NH_3 and NH_4^+-producing fertilizers, organic acids produced from crop residues and litter decomposition, and the addition of wet and dry atmospheric deposition, especially near the point sources (Tabatabai, 1985). Acid precipitation resulting from the combustion of fosil fuels is a widespread phenomenon that acidifies soils in central Europe (Zelles et al., 1987b). Increased acidification is not desired because it lead to the release and leaching of nutrients and mobilization of toxic Al^{3+} in soils. Bacterial populations are decreased and fungi populations are increased by soil acidification, and the whole soil microbial community structure is also altered (Abrahamsen et al., 1980; Alexander, 1980; Baath et al., 1980). Soil quality is affected by acidification, because microorganisms play a key role in the turnover of organic matter and minerals in soils, which, in turn, is very important to soil development and plant growth. Adverse effects to the soil microbial populations by soil acidification are correlated to the changes that occur to soil process such as nitrification, denitrification, and glucose mineralization (Harmsen and van Schreven, 1955; Tabatabai, 1985).

Lime, ash fertilization, and prescribed burning are practices that are used to increase the acid neutralizing capacity of soils. Application of lime to soils as limestone ($CaCO_3$) and dolomite (Ca-Mg CO_3) are the most common practice in the United States and some areas relies on the addition of lime coupled with fertilizers for the production of adequate crop yields and maintenance of soil quality. Coal combustion residues have been also shown to be useful liming

materials (McCarty et al. 1994). The addition of lime materials increases the soil pH, and causes significant changes on the chemical and biochemical reactions and in microbiological processes, with modifications in the solubility of many chemical compounds (Naftel, 1965).

Recent studies have focused on the problem of acidification of forest soils, because these systems have not been as well studied and managed as their agricultural counterparts (Zelles et al., 1987a, 1990; Wanner et al., 1994). Long term field experiments with repeated lime practices cause increases in the soil organic matter in the humus layer of Norway Spruce stands (Derome et al., 1986; Derome and Patila , 1990; Derome, 1990, 1991). Badalucco et al., (1992) reported their findings on the effect of liming on some chemical, biochemical, and microbiological properties of acid soils under spruce. In that study, liming soils with pulverized $CaCO_3$, added on the basis of the lime requirement estimated by the Schoemaker–MacLean-Pratt buffer method (Schoemaker et al., 1962), caused an increase on the cumulative CO_2 evolution, the microbial biomass C and N values, and the activity of dehydrogenase in the soil. Others have also reported increases in the microbial biomass and activity upon liming (Zelles et al., 1987, 1990; Badalucco et al., 1992). Zelles et al. (1990) demonstrated that there are changes in the microbial communities structure by soil liming, and showed that fungi populations are decreased upon liming, because they are unable to function optimally at the higher pH developed because their ecological role is taken over by the prokaryotes. These shifts in the type of soil

microorganisms, that are predominant at different soil pH, significantly affect the level and type of enzyme activities in soils.

Studies of liming soils have focused mostly on the changes of the activity of acid phosphatases in forest soils, because of the positive correlation between phosphate availability and soil pH (Haynes and Swift, 1988; Cepeda et al., 1991; Illmer and Scchinner, 1991). Phosphatase synthesis is repressed when available P concentrations are increased in soil (Nannipieri et al., 1978). Others have reported the decreases of phosphatase activity are not related to an increase in the available P (Haynes and Swift, 1988; Badalucco et al., 1992). Studies on the effect of liming on the activities of different enzymes in agricultural soils are needed to obtain a better assessment of the effects of such practices on the status of C, N, P, and S cycling in soils.

Tillage and Residue Management Effects on Enzyme Activities in Soil

Tillage and crop-residue management practices may lead to significant changes in biological, chemical, and microbiological properties, including the biochemical reactions in soils. Previous research has shown that no-till reduces soil erosion and water evaporation, and increases water infiltration and soil organic-matter levels (Doran 1980a,b; Tracy et al., 1990). Studies have also shown there is organic C and N accumulated at the soil surface (0 –2.5 cm) under reduced tillage involving crop residue placement (Havlin et al., 1990). Accumulation of soil nutrients at the soil surface such as S and P, due to no-

tillage practices with residue placement, has been also reported (Tracy et al., 1990). The use of such management practices will also alter the composition, distribution, and activities of soil microbial communities and soil enzymes (Doran, 1980a,b; Dick, 1984; Magnan and Lynch, 1986). Dick (1984) and Kandeler et al. (1999) reported about the potential of measuring soil enzyme activities as early indicators of changes in soil properties induced by tillage practices. Studies have shown the microbial biomass and soil enzyme activities are greater in surface no-till soils than in plowed soils, while the reverse trend is observed in deeper layers (Angers et al., 1993; Kandeler and Bohm, 1996; Kandeler et al., 1999). Dick (1984) reported the activities of acid phosphatase, alkaline phosphatase, arylsulfatase, invertase, amidase, and urease in 0-7.5 cm surface soils were significantly greater in soils from no-till plots as compared with those from conventional tillage plots. Jordan et al. (1995) reported higher activities of alkaline and acid phosphatases in continuous corn under no-till than in continuous corn under conventional tillage. Many researchers have also reported the activities of phosphatases are generally greater in soils under no-till than in soils under conventional tillage (Doran, 1980b; Angers et al., 1993). Studies by Kandeler et al. (1999) with different tillage treatments applied to the top 10 cm of soil showed that the activities of alkaline phosphatase, xylanase, and protease responded faster than other tests (e.g., nitrification, N mineralization). The activities of the enzymes showed the following pattern of response to the different tillage practices used: minimum > reduced >

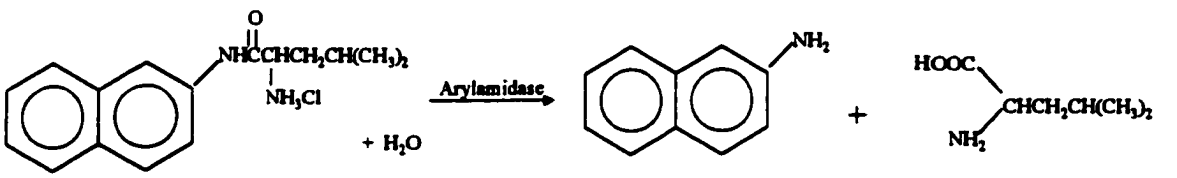
conventional tillage. Studies with Iowa soils showed the activities of the glycosidases (α- and β- glucosidase and α- and β- galactosidase), amidohydrolases (urease, amidase, L-asparaginase, and L-glutaminase), phosphatases (alkaline and acid phosphatases, phosphodiesterase and inorganic pyrophosphatase), and arylsulfatase were significantly affected by tillage and residue placement (Deng and Tabatabai, 1996a,b; 1997). Results from such work showed the activities of all the enzymes studied were greater in no-till/double mulch treatments than in the other treatments, including no-till/bare, no-till/normal, chisel/normal, chisel/mulch, moldboard/normal, and moldboard/mulch.

PART I. ASSAY OF ARYLAMIDASE ACTIVITY IN SOILS

INTRODUCTION

The enzyme amino acid arylamidase [α-aminoacyl-peptide hydrolase (microsomal) EC 3.4.11.2] catalyzes the hydrolysis of an N-terminal amino acid from peptides, amides or arylamides. Arylamidase is widely distributed in the tissues and body fluids of all animals (Hiwada et al., 1980), plants, and microorganisms (Appel, 1974). The chemical nature of N in soils is such that a large proportion (15-25%) of organic N is often released as NH_4^+ by 6 *M* HCl hydrolysis. The information available suggests that a portion of the released NH_4^+ is derived from amino acid residues in linear amides and arylamides of soil organic matter (Sowden, 1958). Several papers have been published on linear amidase in soils and on the enzyme involved in hydrolysis of amides and amino acids such as asparagine, aspartic acid, and glutamine (Frankenberger and Tabatabai, 1980, 199a,b; Senwo and Tabatabai, 1996), but the possibility of the presence of arylamidase in soils has not been explored. The activity of this enzyme in soils deserves investigation because present knowledge indicates that a variety of arylamides are present in soils (Stevenson, 1994). Arylamidase may play an important role as an initial limiting step in mineralization of organic N in soils. Thus, understanding the environmental controls on the activity of this enzyme in soil is important for better understanding the N cycling process. This enzyme is capable of hydrolyzing the neutral amino acids β-naphthylamides and

p-nitroanilides according to the following reaction (using the amino acid L-leucine as an example):



L-leucine β-Naphthylamide β-Naphthylamine Leucine

The objectives of the present work were (i) to develop a simple and sensitive method for the assay of arylamidase in soils and to ascertain the factors affecting the observed activity and (ii) to determine the kinetic parameters of the reaction catalyzed by this enzyme (i.e, K_m and V_{max} values), activation energy (E_a) , enthalpy of activation (ΔH_a), and temperature coefficients(Q_{10}). The method developed involves colorimetric determination of the β-naphthylamine produced by arylamidase activity when soil is incubated with 0.1 *M* THAM buffer (pH 8.0) and L- leucine β-naphthylamide hydrochloride at 37°C for 1 h.

MATERIAL AND METHODS

Soils and their Properties

The soils (Table 1) used were surface samples (0-15 cm) selected to provide a wide range of chemical and physical soil properties. In the properties reported in Table 1, pH was determined by a combination glass electrode (soil:water or soil:0.01*M* $CaCl_2$ ratio, 1:2.5), organic C by the Mebius method (1960), total N by the semimicro-Kjeldahl procedure (Bremner and Mulvaney, 1982), and particle-size distribution by the pipette method (Kilmer and Alexander, 1949).

Reagents

THAM Buffer (0.1 *M*, pH 5.0 or 6.0) -- Prepared by dissolving 2.44 g of tris (hydroxymethyl) aminomethane (THAM buffer, Fisher Scientific Co., Chicago) and 2.72 g of sodium acetate in about 50 mL of water. Thus, the buffer was also made 0.1 *M* with respect to $CH_3COONa \cdot 3\ H_2O$. The pH of the buffer was adjusted by titration to pH 7 with the addition of 1 *M* CH_3COOH. Further adjustments to pH 5 or 6 were accomplished with approximately 0.2 *M* HCl. The solution was diluted to 200 mL with water.

THAM Buffer (0.1 *M*, pH 7.0) -- Prepared by dissolving 2.44 g of tris (hydroxymethyl) aminomethane (THAM buffer, Fisher Scientific Co., Chicago) and 2.72 g of $CH_3COONa \cdot 3\ H_2O$ in about 50 mL of water. Thus, the buffer was also made 0.1 *M* with respect to sodium acetate. The pH of the buffer was

Table 1. Selected properties of the soils used

Soil		pH[a]					
Series	Subgroup	H_2O	$CaCl_2$	Org. C	Total N	Clay	Sand
				g kg^{-1}			
Downs	Mollic Hapludalf	5.1	4.3	14.3	1.38	163	53
Grundy	Aquic Argiudoll	6.3	5.2	15.4	1.09	300	11
Clinton	Typic Hapludalf	6.1	5.7	16.4	1.46	318	15
Monona	Typic Hapludoll	5.7	5.1	25.4	2.54	210	58
Clarion	Typic Hapludoll	5.4	4.7	27.8	2.39	196	384
Webster	Typic Haplaquoll	6.9	6.5	32.4	3.80	280	302
Harps	Typic Calciaquoll	7.9	7.5	44.0	2.43	356	188

[a] Soil:water or soil: 0.01 *M* $CaCl_2$ ratio, 1:2.5.

adjusted by titration to pH 7 with the addition of 1 *M* acetic acid, and it was diluted to 200 mL with water.

THAM Buffer (0.1 *M*, pH 8.0, 9.0 and 10.0) -- Prepared by dissolving 2.44 g of tris (hydroxymethyl) aminomethane (THAM buffer, Fisher Scientific Co., Chicago) in about 50 mL of water, adjusting the pH by titration with approximately 0.05 *M* H_2SO_4, and diluting the solution to 200 mL with water.

L-Leucine β-Naphthylamide Solution (8.0 m*M*) -- Prepared by dissolving 0.2342 g of hydrochloride salt of L-leucine β-naphthylamide (Sigma Chemical Co., St. Louis, MO) in water and adjusting the volume to 100 mL with water.

Ethanol -- (95%).

Acidified Ethanol (0.26 *M* HCl) -- Prepared by adding 4.32 mL of concentrated HCl to ethanol and adjusting the volume to 200 mL with ethanol.

p-Dimethylaminocinnamaldehyde Solution (0.6 mg mL^{-1}; Sigma Chemical Co., St. Louis, MO) -- Prepared by dissolving 0.12 g of *p*-dimethylaminocinnamaldehyde in ethanol and adjusting the volume to 200 mL with ethanol.

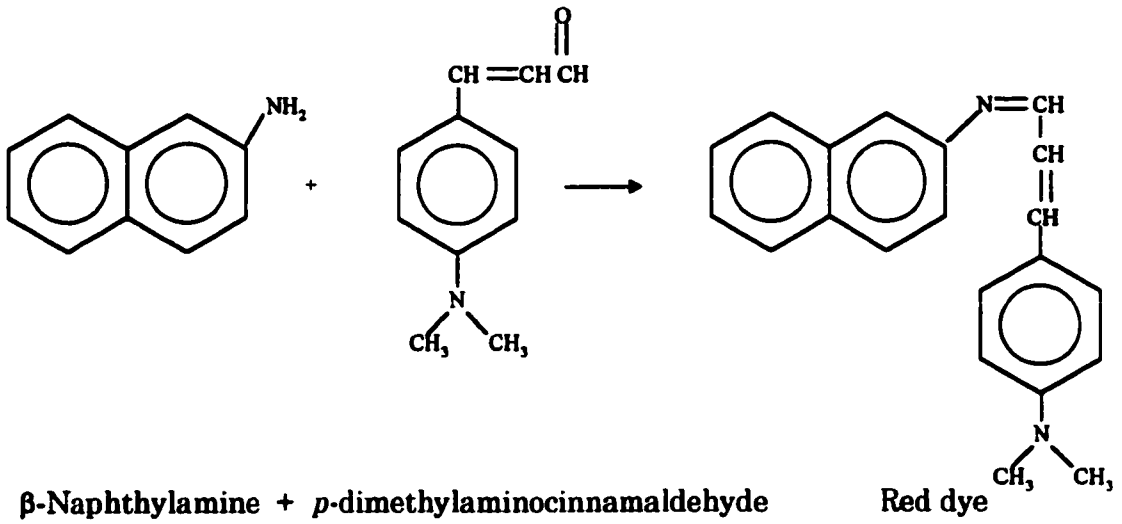
Standard β-Naphthylamine Stock Solution (125 μg mL^{-1}) -- Prepared by dissolving 12.5 mg of β-naphthylamine (Sigma Chemical Co., St. Louis, MO) in 75 mL deionized water containing 5 mL of ethanol in a 100-mL volumetric flask, and adjusting the volume with deionized water.

Standard β-Naphthylamine Working Solutions -- Prepared by transferring 1, 2, 3, 4, 5, or 6 mL of the standard β-naphthylamine stock solution

(125 μg mL^{-1}) into a 25- mL volumetric flask, and adjusting the volume with deionized water. These standard solutions contain 5, 10, 15, 20, 25, or 30 μg of β-naphthylamine mL^{-1}, respectively.

Assay Procedure

A 1-g soil sample (air-dried, <2mm) in a 25-mL Erlenmeyer flask was treated with 3 mL of 0.1 *M* THAM buffer (pH 8.0) and 1 mL of 8.0 *mM* L-leucine β-naphthylamide hydrochloride. The flask was swirled for a few seconds to mix the contents and was stoppered and placed on a shaker in an incubator (37 °C) for 1 h. After incubation, the reaction was terminated by adding 6 mL of ethanol (95%). The soil suspension was immediately mixed and transferred into a centrifuge tube and centrifuged for 1 min at 17,000 *g*. The supernatant was transferred to a test tube to prevent any further hydrolysis of the substrate, and a 1-mL aliquot of this supernatant was treated (in a second test tube) with 1 mL of ethanol, 2 mL of acidified ethanol, and 2 mL of the *p*-dimethylaminocinnamaldehyde reagent. The solution was mixed on a vortex mixer, after adding each of the reagents. The intensity of the resulting red azo compound was measured at 540 nm (Hiwada et al., 1977). The reaction involved is as follows:



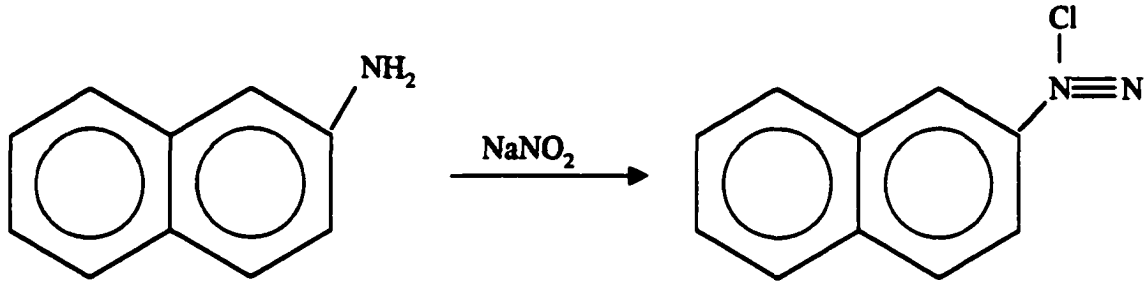
β-Naphthylamine + *p*-dimethylaminocinnamaldehyde Red dye

In the assay method, when the color intensity of the red azo compound exceeded the concentration of the highest β-naphthylamine standard, an aliquot of the red azo compound was diluted with ethanol until the reading was within the limits of the calibration graph. The calibration graph was prepared by treating 1 mL of each of the standard working solutions in a test tube with 1 mL of ethanol, 2 mL of the acidified ethanol, and 2 mL of reagent containing *p*-dimethylaminocinnamaldehyde.

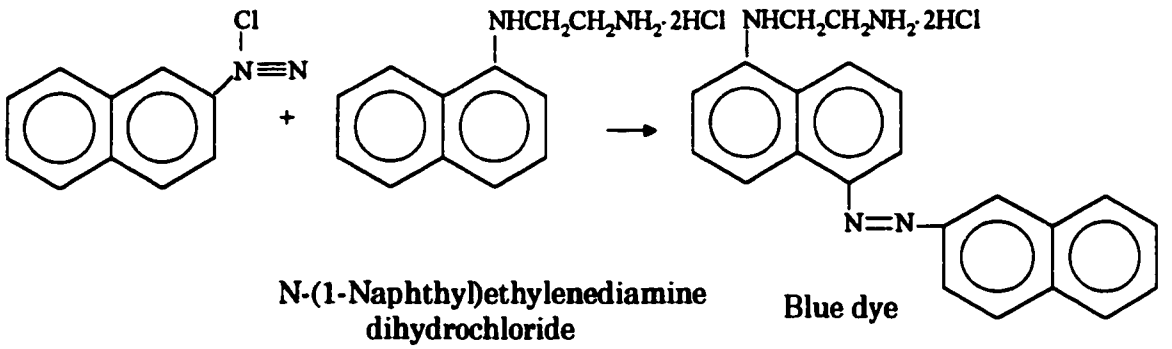
Controls were included as described for the assay, but the 1 mL of the substrate was added after incubation. Subsets of samples were used to study the factors affecting the activity of this enzyme. These were selected to give ranges in the activity values and to avoid overlapping of the curves obtained. Unless otherwise indicated, all results reported are averages of duplicate assays

on air-dried soils and are expressed on a moisture-free basis. Moisture was determined from the loss in weight after drying at 105°C for 36 h. At all data points reported in the figures, the differences between the duplicate values were smaller than the point size.

An alternative method is available for colorimetric determination of the β-naphthylamine produced (Goldbarg and Rutenburg, 1958). This method involves diazotization of the β-naphthylamine released with $NaNO_2$, decomposition of the excess $NaNO_2$ with ammonium sulfamate, and conversion of β-naphthylamine to a blue azo compound at pH 1.2 with N-(1-naphthyl) ethylenediamine dihydrochloride solution. The absorbance of the blue azo compound is measured at 700 nm. The reactions involved are as follows:



β-Naphthylamine



This method, however, is complicated and tedious. Therefore, we evaluated the optimal conditions for development of the red azo compound. The color of the red azo compound produced from the reaction described is stable for at least 24h.

Experiments

To determining the optimal conditions to assay the activity of arylamidase in soils, the factors studied included buffer pH, substrate concentration, amount of soil, time of incubation, temperature of incubation, air-drying of field-moist soils, preheating temperature, and selected inhibitors. The results obtained in the experiment on the effect of substrate concentration on arylamidase activity in soils were used to calculate the K_m and V_{max} values. The values were calculated using the three possible linear transformations of the Michaelis-Menten equation: Lineweaver-Burk plot, Eadie-Hofstee plot, and Hanes-Woolf plot. The parameters of energy of activation (E_a), enthalpy of activation (ΔH_a) and temperature coefficients (Q_{10}) were calculated from the arylamidase activity

values obtained in the experiment on the effect of temperatures on the activity of the enzyme. The results obtained will be discussed under subheadings according to the factors studied.

RESULTS AND DISCUSSION

Buffer pH

To ascertain the optimal pH for arylamidase activity in soils, the activity was assayed at 2 m*M* of L-leucine β-naphthylamide in the presence of THAM buffer at pH values ranging from 5 to 10. Because of the limiting buffer strength of THAM with pH values < 7, the buffer solutions at pH 5 and 6 were also made to be 0.10 *M* with respect to $CH_3COONa \cdot 3H_2O$ and the pH was adjusted to 7 with the addition of 1 *M* CH_3COOH. Further adjustment of the pH 5 or 6 was made with the addition of 0.2 *M* HCl. The rate of the β-naphthylamine produced was optimal at buffer pH = 8.0 (Figure 1). This value is in agreement with the range (6.1-8.0) reported by Appel (1974) for this enzyme purified from human and animal organs, plants, and microorganisms. The agreement of the optimal pH value (8.0) with the upper limit, but not with the lower limit, reported for this enzyme in other biological materials is expected, because it is well known that the pH optima of enzymes in solutions are about 1.5 pH units lower than the same enzyme in soils (McLaren and Estermann, 1957). A shift in pH optimum to higher values in solution occurs because the Bronsted acidity at the clay surface is significantly greater than in the bulk solution (Boyd and Mortland, 1990).

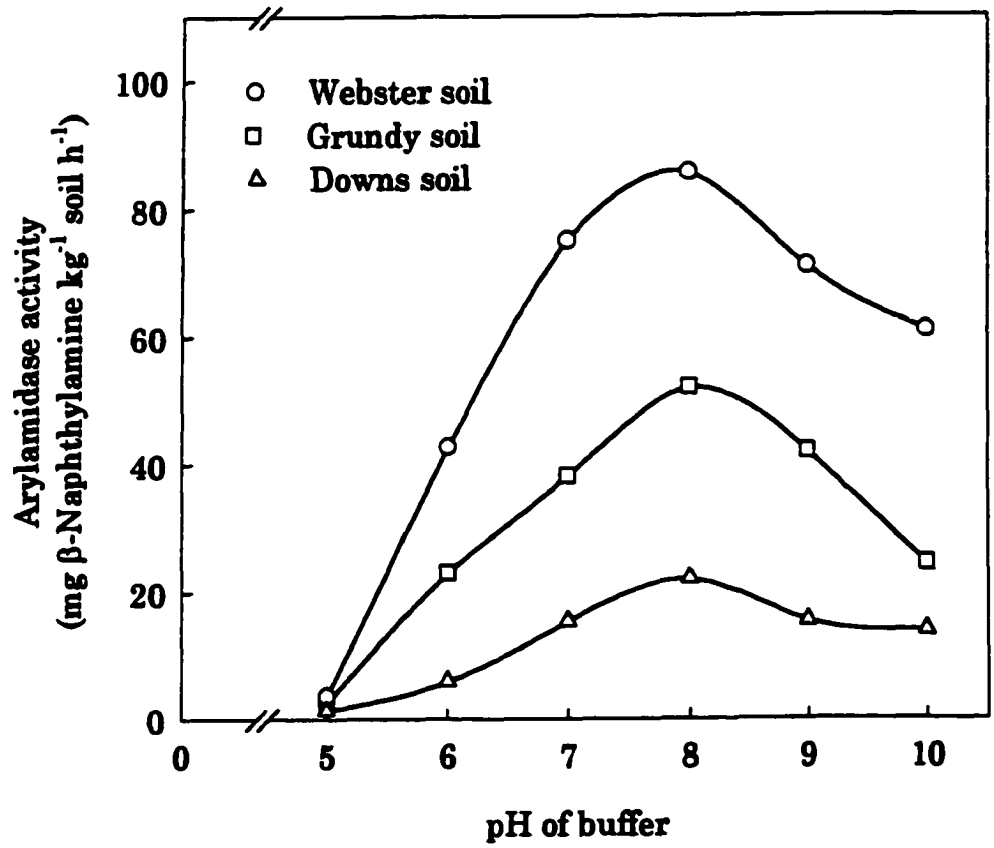


Figure 1. Effect of buffer pH on release of β-naphtylamine in assay of arylamidase activity in soils

Substrate Concentration and Amount of Soil

The initial rates of arylamidase activity in soils were measured at various substrate concentrations (Figure 2). The reaction velocity increased with increases in the substrate concentration, and showed that the concentration (2 m*M*) adopted was satisfactory for the assay of arylamidase activity in soils. At this concentration, the reaction essentially followed zero-order kinetics. The rate of the reaction at this substrate concentration was, therefore, dependent on the enzyme concentration in the 1 g of soil used, evident from the levels of activity of the soils shown in Figure 2.

Studies on the specificity of arylamidase showed that, in addition to L-leucine β-naphthylamide, this enzyme hydrolyzes L-alanine β-naphthylamide. Results reported in Table 2 show that, with the exception of the activity of the Harps soil, both substrates gave similar activity values.

A linear relationship between the amount of soil and the amount of β-naphthylamine produced showed that 1 g of soil was satisfactory for assaying the activity of this enzyme (data not shown). This is further evidence that the procedure described measures arylamidase activity and that neither the substrate concentration nor the products released influence the reaction rate of this enzyme in soils.

Procedure to Terminate the Reaction after Incubation

The proper conditions to stop the reaction of the hydrolysis of the amino acid β-naphthylamide upon incubation of soil samples were evaluated for this

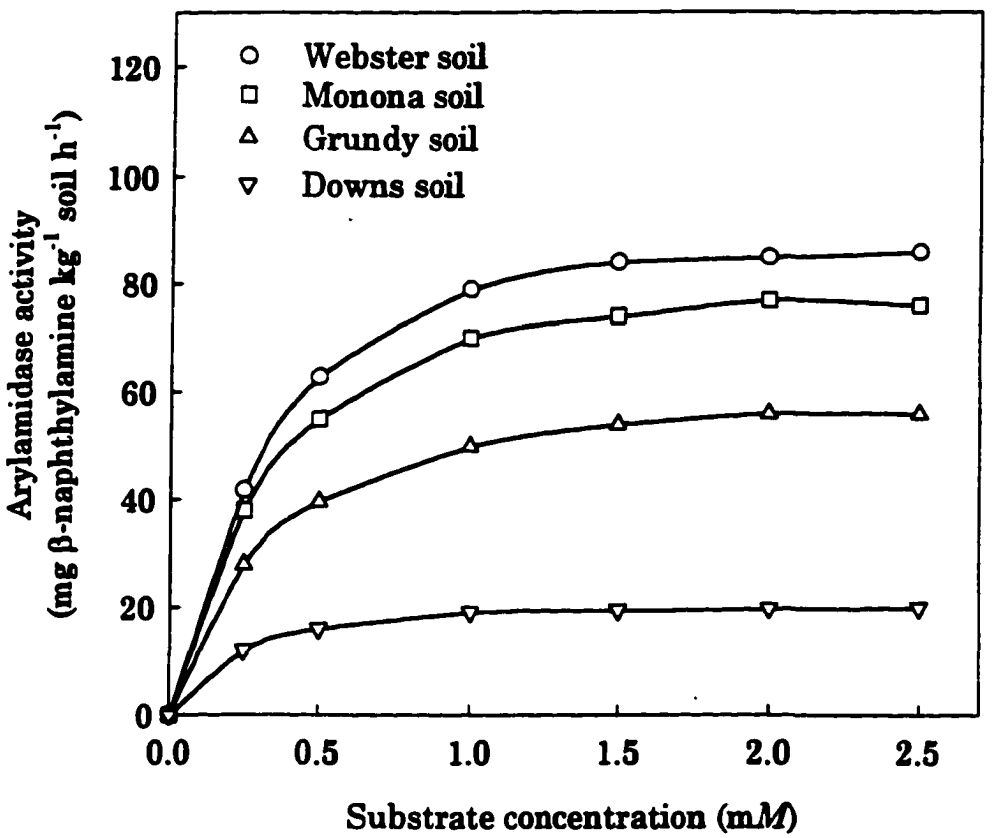


Figure 2. Effect of substrate concentration on release of β-naphthylamine in assay of arylamidase activity in soils

Table 2. Specificity of arylamidase in soils towards its substrates

Soil	Field-moist soil		Air-dried soil	
	L-Leucine [a]	L-Alanine [a]	L-Leucine	L-Alanine
	mg β–Naphthylamine kg^{-1} soil h^{-1}			
Harps	135	246	135	132
Monona	73	98	88	78
Webster	89	93	87	88
Grundy	55	54	57	61
Clinton	48	60	63	64
Clarion	40	65	46	48
Downs	19	19	19	24

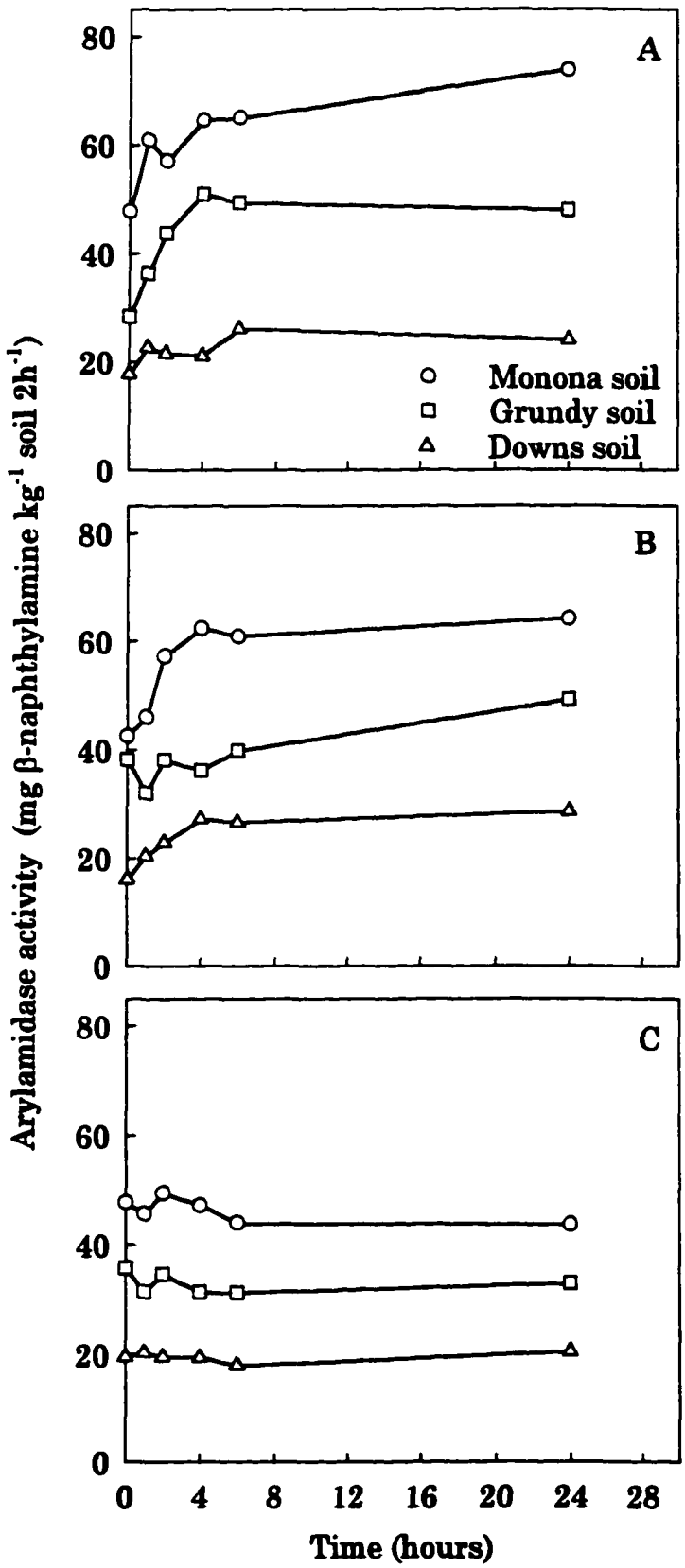
[a] Amino acid attached to naphthylamine at the β-position.

enzyme assay method. Figure 3A shows that arylamidase activity was not completely stopped in the Monona, Grundy and Downs soils with the addition of 6 mL of ethanol (95%). Ethanol was used to extract the product β-naphthylamine; it also denatures the enzyme protein. However, determination of the enzyme activity over time showed that the enzyme was somewhat active up to 8 h after the addition of ethanol or with addition of ethanol and centrifugation of the samples (Figure 3B). But, the addition of ethanol, centrifugation, and the transfer of the supernatant to a test tube was more effective in stopping the arylamidase activity after the incubation of soils (37°C).

Time and Temperature of Incubation

The relationship between the amount of product formed and the time of incubation is usually linear in enzyme-catalyzed reactions in soils, as long as the enzyme is stable and retains it full activity (Frankenberger and Tabatabai, 1980). The observed linear relationship indicated that the method developed is not complicated by microbial growth or assimilation of enzymatic products by microorganisms (Figure 4). Formation of β-naphthylamine in the soils studied was a zero-order reaction for at least 4 h of incubation. The incubation time used (1 h) allowed ample time for accumulation of β-naphthylamine. It is not necessary to incubate the soil-substrate-buffer mixture for 4 h because results reported in Figure 4 show that a much shorter incubation time can be adopted (e.g., 1 h).

Figure 3. Effect of different procedures to terminate the reaction of arylamidase activity in soils, addition of ethanol (A), addition of ethanol and centrifugation (B), and addition of ethanol, centrifugation, and transferring the supernatant to another test tube (C)


A
B
C
Monona soil
Grundy soil
Downs soil
Arylamidase activity (mg β-naphthylamine kg-1 soil 2h-1)
Time (hours)
0
20
40
60
80
0
4
8
12
16
20
24
28

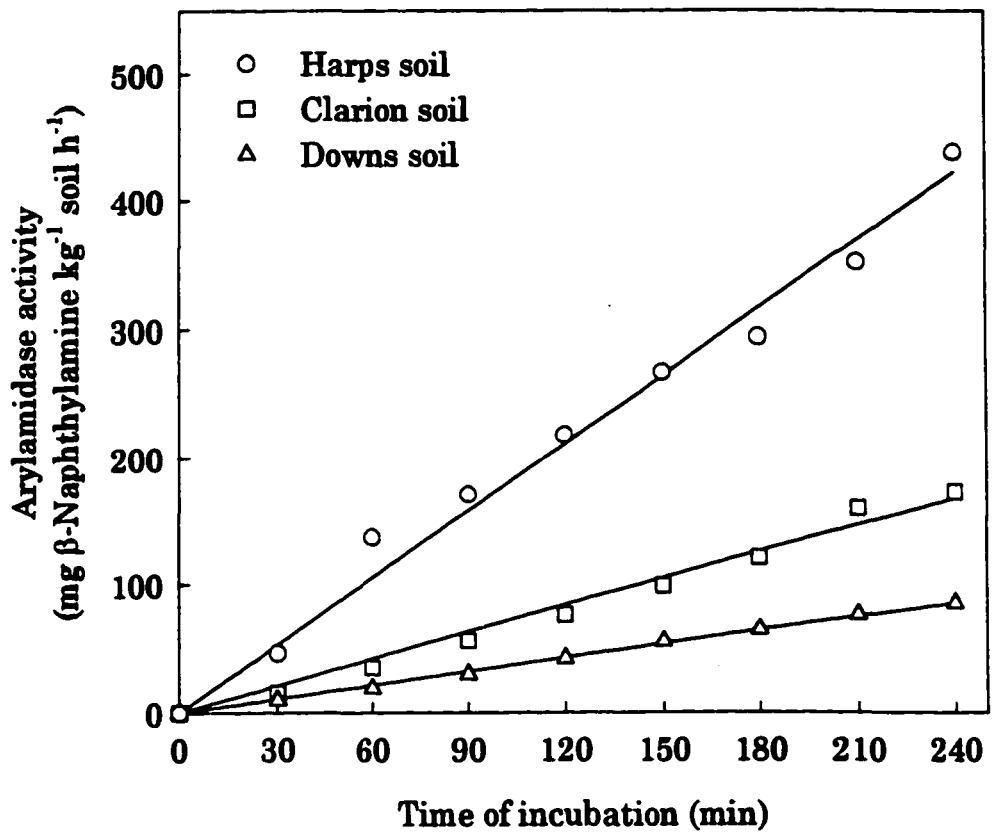


Figure 4. Effect of time of incubation on release of β-naphthylamine in assay of arylamidase activity in soils

In general, enzyme-catalyzed reactions proceed at faster rates with increasing temperature up to a temperature above which the enzyme activity decreases due to denaturation. Optimal arylamidase activity occurred at 60°C under the assay conditions described (Figure 5). Denaturation occurred at temperatures > 60°C for both field-moist and air-dried soils, which is similar to those of asparaginase (Frankenberger and Tabatabai, 1991b), amidase (Frankenberger and Tabatabai, 1980), arylsulfatase (Tabatabai and Bremner, 1970), rhodanese (Tabatabai and Singh, 1976), and phosphodiesterase (Browman and Tabatabai, 1978) activities in soils. In general, the temperature needed to inactivate an enzyme in soil is about 10°C greater than the temperature needed to inactivate the same enzyme in the absence of soil (Skujins, 1967).

The activity of arylamidase was assayed at 37°C because this temperature has been used extensively for assay of other enzymes in many biological materials, including soils (Tabatabai, 1994), and because preliminary tests indicated that at temperatures < 30°C, it could be difficult to quantitatively detect the low levels of activity exhibited by some soils, especially sandy soils. Tests with other soils showed that it was not necessary to use a higher temperature to obtain precise results under the conditions of the assay method described (Figure 5).

The temperature used in drying field-moist soils and storing air-dried samples affect the enzyme activities (Tabatabai, 1994). The effect of

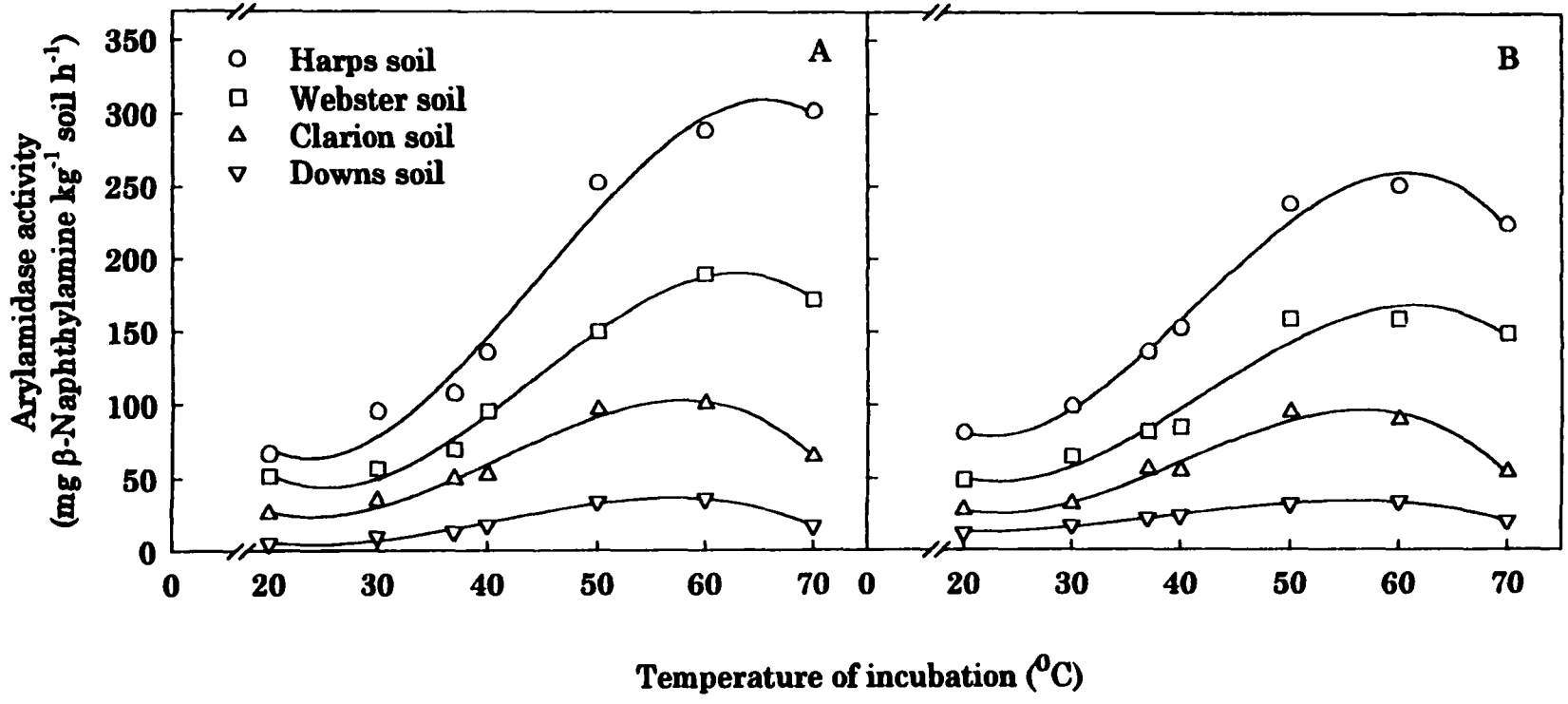


Figure 5. Effect of incubation temperature on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Field-moist soils; (B) air-dried soils

temperature on the stability of arylamidase in field-moist and air-dried soils has not been studied. In this work, the soil samples were exposed to temperatures ranging from 20 to 120°C for 2 h, and the arylamidase activity was assayed at 37°C. Results showed that the activity of this enzyme was stable up to 40°C in field-moist soils and up to 60°C in air-dried soils (Figure 6), suggesting that air drying of field-moist soil samples contributes to the stabilization of the enzyme protein. The rate of inactivation of this enzyme by temperature was much faster in field-moist than in air-dried soils. Arylamidase activity was completely destroyed in field-moist soils heated at 110-120°C, but the air-dried soils contained residual activity after heating at 120°C for 2 h.

In calculating the activation energy of soil enzymes, it is assumed that the incubation temperature (20-60°C for arylamidase, Figure 5) has no effect on the stability of the enzyme. The results reported in Figure 6B support the validity of this assumption.

Activation Energy, Enthalpy of Activation, and Kinetic Parameters

Temperature dependence of enzyme-catalyzed reactions is well documented. The dependence of the rate constant on temperature (below the inactivation temperature) of an enzyme-catalyzed reaction can be represented by the Arrhenius equation:

$$k = A \exp(-E_a/RT),$$

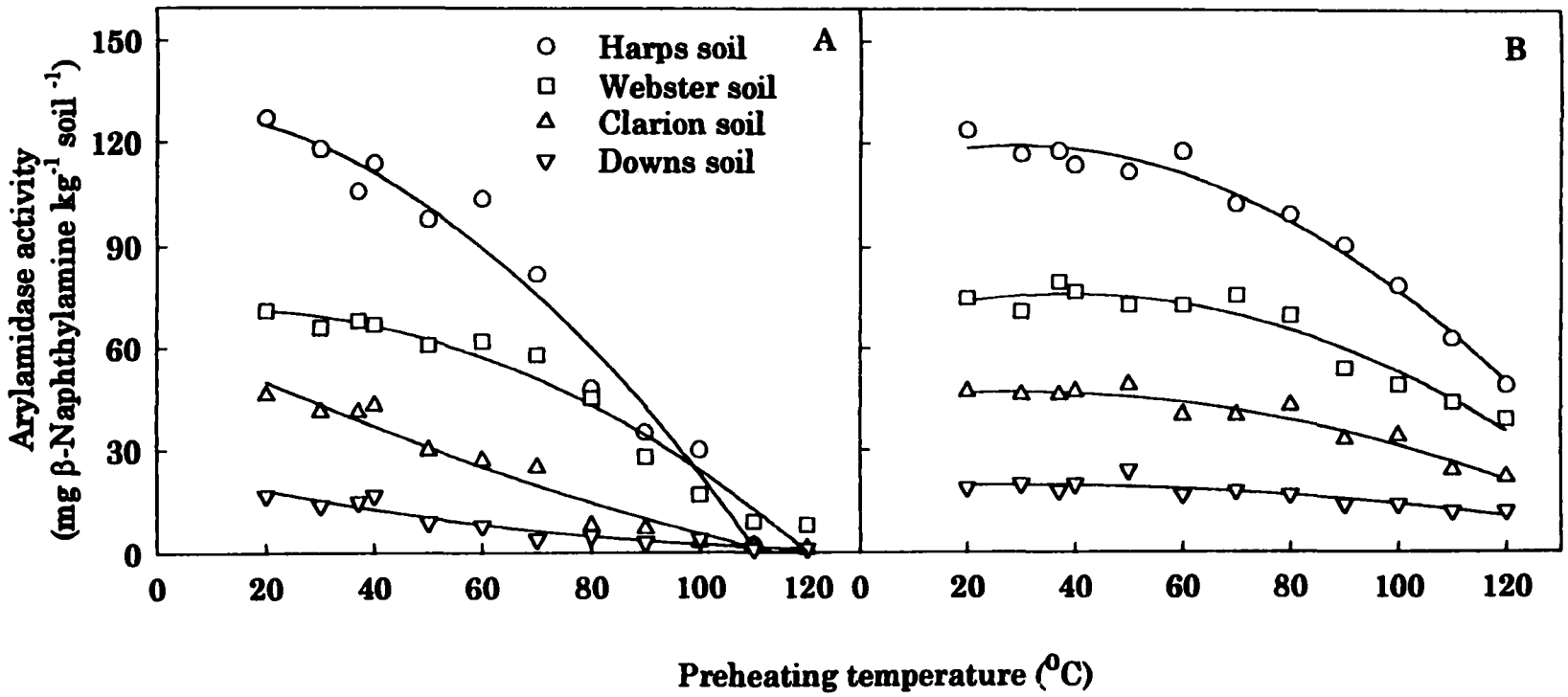


Figure 6. Effect of preheating temperature on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Field-moist soils; (B) air-dried soils

where k is the rate constant, A is the pre-exponential factor, E_a is the activation energy, R is the gas constant, and T is the absolute temperature in °K. The logarithmic transformation of the Arrhenius equation is expressed as follows:

$$\log k = (-E_a/2.303RT) + \log A.$$

The activation energy can be calculated from a plot of log k (or apparent values or any parameter that is proportional to the rate constant) against $1/T$ (Segel, 1975, p. 932). The Arrhenius equation plot for arylamidase activity in the soils studied was linear between 20 and 50°C (Figure 7). The activation energies of the enzyme reaction in the soils were obtained from the slope, and the values for field-moist and air-dried soils ranged from 30.6 to 49.8 kJ mol^{-1} and from 26.2 to 32.4 kJ mol^{-1}, respectively (Table 3). These values are within the ranges reported for other soil enzymes (Tabatabai, 1994). The means of Q_{10} values for arylamidase in four soils for temperatures between 20 and 40°C ranged from 1.32 to 1.71 (overall avg. = 1.44). These values are within the ranges reported for other soil enzymes, which are normally < 2 (Tabatabai, 1994).

The enthalpy of activation was calculated from the slope of a plot of log k/T (k = apparent values or any parameter that is proportional to the rate constant) against $1/T$ (Figure 8). And it also can be calculated from the theoretical equation:

$$E_a = \Delta H_a - RT$$

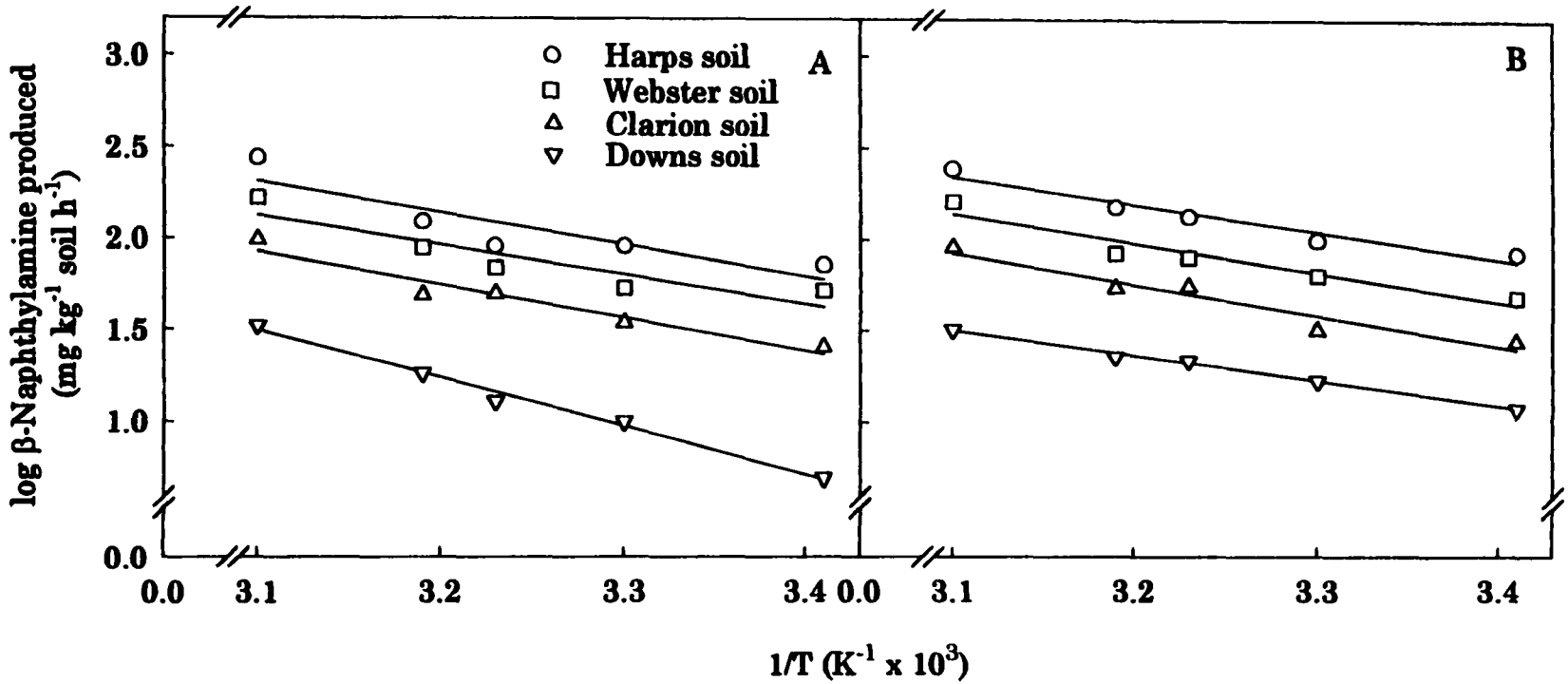


Figure 7. Arrhenius equation plot of arylamidase activity in soils. (A) Field-moist soils; (B) air dried soils (T = absolute temperature)

Table 3. Activation energies (E_a) and temperature coefficient values of arylamidase activity in soils

Soil [b]	E_a	ΔH	Q_{10} of temperature (°C) indicated [a]		
			30	40	Mean
	kJ mol^{-1}	kJ mol^{-1}			
Harps					
FM	32.9	29.8	1.39	1.27	1.33
AD	28.3	25.9	1.22	1.55	1.39
Webster					
FM	30.6	27.5	1.15	1.65	1.40
AD	30.3	27.8	1.33	1.31	1.32
Clarion					
FM	34.5	31.2	1.35	1.40	1.38
AD	32.4	30.1	1.14	1.72	1.43
Downs					
FM	49.8	47.0	2.00	1.42	1.71
AD	26.2	23.7	1.80	1.35	1.58

[a] $Q_{10} = \frac{\text{Arylamidase activity at given temperature}}{\text{Arylamidase activity at given temperature - 10°C}}$.

[b] FM = field-moist soil ; AD = air-dried soil.

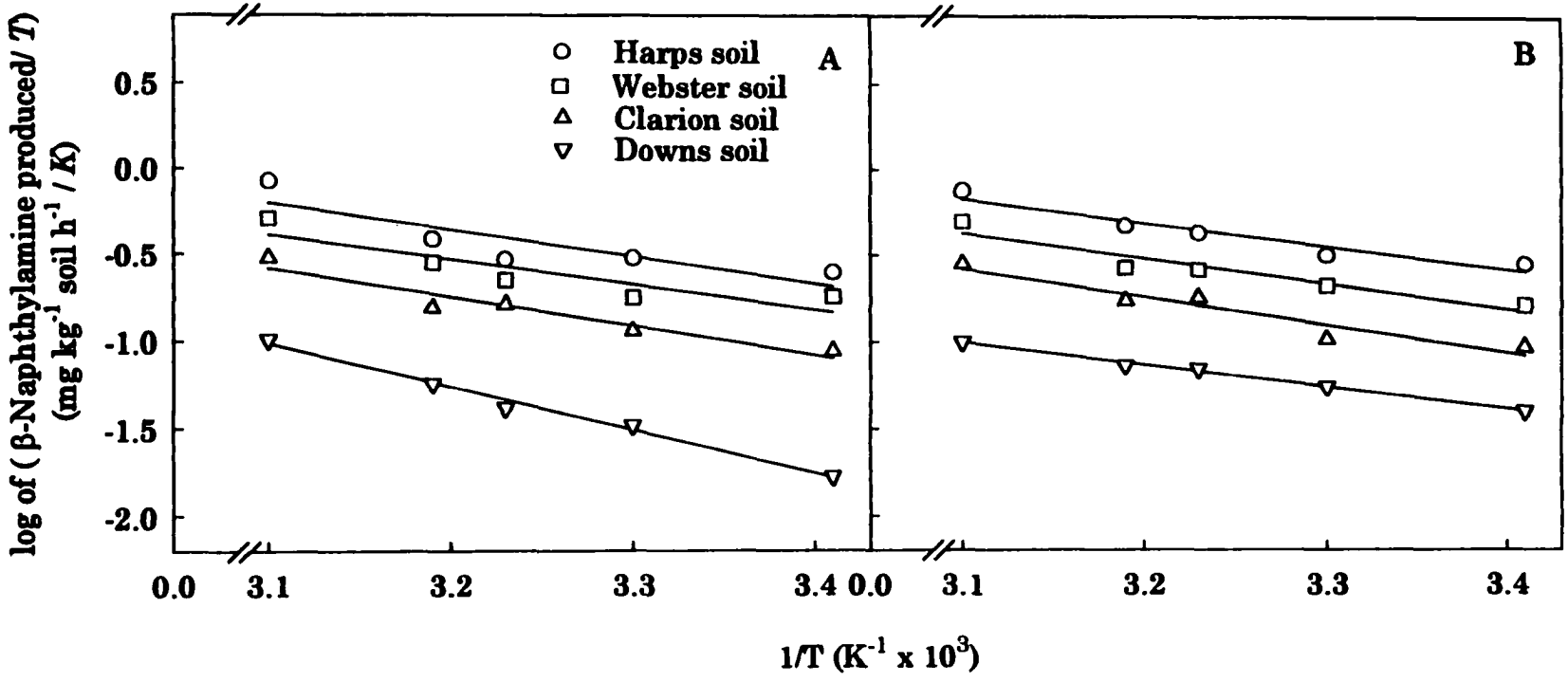
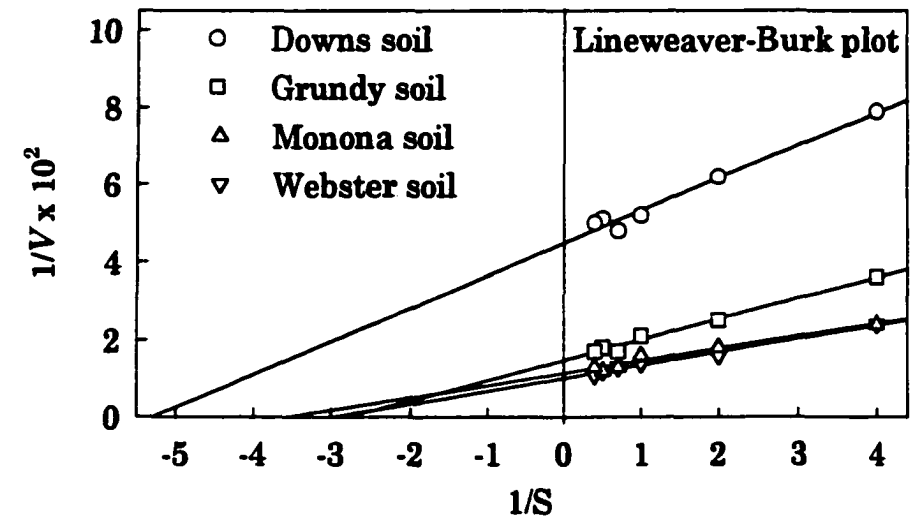


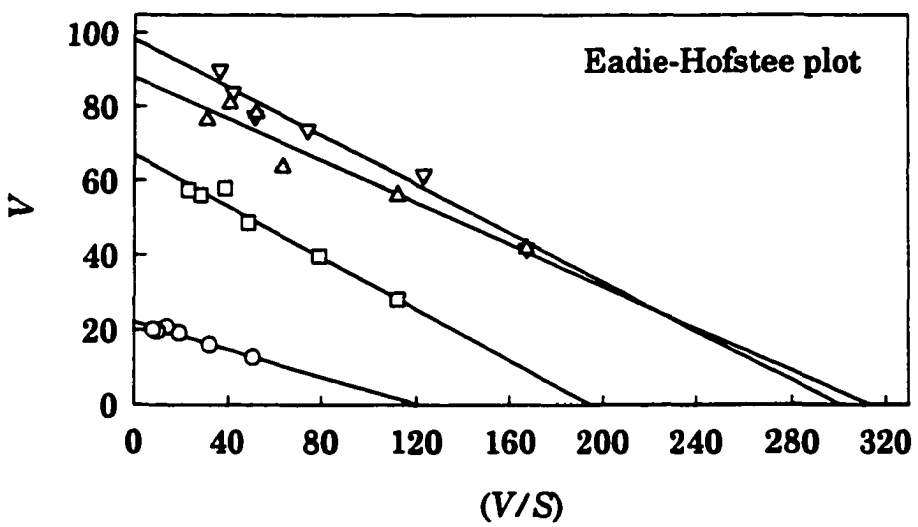
Figure 8. Equation plot of arylamidase activity in soils. (A) Field-moist soils; (B) air dried soils (T = absolute temperature)

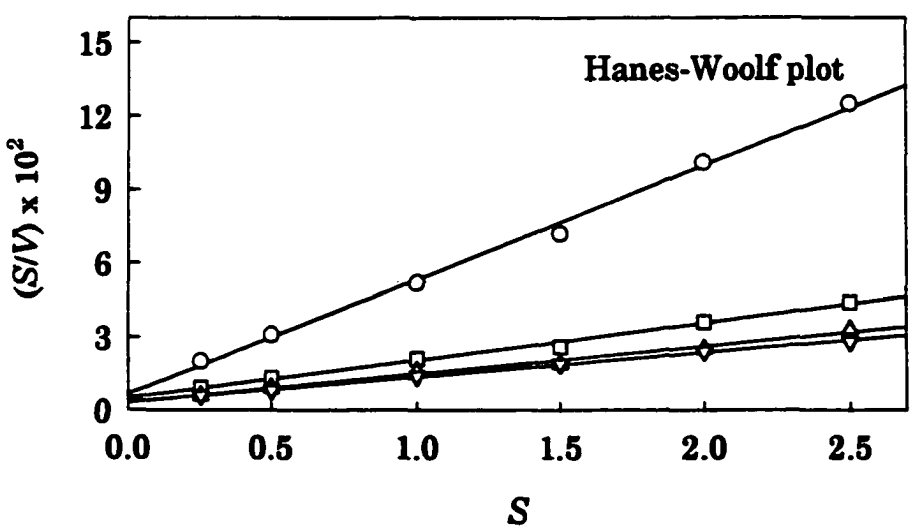
where R is the gas constant and T is temperature in Kelvin. The values of enthalpy of activation for air-dried soils ranged from 23.7 to 30.1 kJ mol^{-1} for Downs and Clarion soil, respectively (Table 3). The equation to calculate the enthalpy of activation showed that the values of ΔH_a decreases by 2.5 KJ mol^{-1} with respect to E_a at 37°C (310°K). Both the values calculated from the slope of the plot of log k/T against $1/T$ and the ones obtained with the equation were the same.

Three transformations of the Michaelis-Menten equation applied to arylamidase activity values obtained as a function of L-leucine β-naphthylamide concentration are shown in Figure 9. The straight lines shown are those calculated by regression analysis. By using the Lineweaver-Burk plot, the K_m values for arylamidase in four soils ranged from 0.19 to 0.35 mM (Table 4), suggesting high affinity of the enzyme for its substrate. These K_m values are lower than those reported (6.7-17.9, avg. = 12.3 for eight soils) for linear amidase in soils by using formamide as a substrate (Frankenberger and Tabatabai, 1980). These values, however, are one order of magnitude greater than those reported (5.5-8.7 x 10^{-2} mM) for arylamidase extracted from several human organs (Hiwada et al., 1977). The greater K_m values of arylamidase activity in soils compared with those reported for protein preparations is the result of enzyme adsorption on the clay-organic matter complexes (Tabatabai, 1994). That is because the interaction between the adsorbed enzyme and its substrate in solution is less than that of the free enzyme. Therefore, greater substrate

Figure 9. The three possible linear plots of the Michaelis-Menten equation for arylamidase activity in soils. Substrate concentration (S) is expressed in m*M* and the reaction velocity in mg β-naphthylamine released kg^{-1} soil h^{-1}


Lineweaver-Burk plot
Downs soil
Grundy soil
Monona soil
Webster soil
1/V x 10²
1/S
10
8
6
4
2
0
-5
-4
-3
-2
-1
0
1
2
3
4


Eadie-Hofstee plot
V
(V/S)
100
80
60
40
20
0
0
40
80
120
160
200
240
280
320


Hanes-Woolf plot
(S/V) x 10²
S
15
12
9
6
3
0
0.0
0.5
1.0
1.5
2.0
2.5

concentration is required for an adsorbed enzyme to achieve the same reaction velocity as that of a free enzyme in solution. The V_{max} values of the corresponding soils ranged from 22 to 100 mg β-naphthylamine kg^{-1} of soil h^{-1} (Table 4). Generally, the three methods of calculation showed similar results. Each transformation gives different weight to errors in the variables (Dowd and Riggs, 1965), and this is reflected in the variation of the estimated K_m and V_{max} values obtained for any soil by using the different plots. A similar observation has been reported for kinetic analysis of the reactions catalyzed by phosphodiesterase in soils (Browman and Tabatabai, 1978) and by linear amidase in soils (Frankenberger and Tabatabai, 1980).

Effect of Various Treatments

Tests were performed to identify possible inhibitors and activators of the reaction catalyzed by arylamidase because such tests would provide useful information about the structural requirements of the enzyme-substrate interaction. Treatment of soils with toluene, formaldehyde, dimethylsulfoxide, $HgCl_2$, or iodoacetic acid inhibited arylamidase activity, and autoclaving completely destroyed the enzyme protein in soils (Table 5). The inhibition observed by treatment of soils with each of the last three inhibitors confirm the finding reported by Marks et al. (1968) that the active sites of this enzyme contain sulfhydryl groups.

Table 4. K_m and V_{max} values of arylamidase activity in soils calculated by using three linear transformations of the Michaelis-Menten equation

Michaelis-Menten transformation	Soil	K_m [a]	V_{max} [a]
Lineweaver-Burk plot *(1/V vs 1/S)*	Downs	0.19	22
	Grundy	0.35	67
	Monona	0.29	91
	Webster	0.34	100
Eadie-Hofstee plot *(V vs. V/S)*	Downs	0.18	22
	Grundy	0.34	67
	Monona	0.28	88
	Webster	0.33	98
Hanes -Woolf plot *(S/V vs. S)*	Downs	0.14	21
	Grundy	0.31	67
	Monona	0.29	91
	Webster	0.36	100

[a] K_m is expressed in mM and V_{max} is expressed in mg β-napthylamine produced kg^{-1} soil h^{-1}.

Table 5 . Effects of various treatments on arylamidase activity in soils

Soil treatment	Arylamidase activity in soil specified		
	Downs	Webster	Clarion
	— mg β-Napthylamine kg^{-1} soil h^{-1} —		
Buffer, with toluene	10	40	23
Buffer, without toluene	20	85	45
Unbuffered, with toluene	0	28	4
Unbuffered, without toluene	2	40	4
Formaldehyde (0.3ml)	1	0	1
Dimethylsulfoxide (0.3ml)	5	39	11
$HgCl_2$ (1m*M*)[a]	8	62	26
Iodoacetic acid (1m*M*)[a]	11	65	30
Autoclaving[b]	0	0	0

[a] Reagent was added dissolved in 0.1 *M* THAM buffer (pH 8.0) for assay of arylamidase activity.

[b] Before incubation.

Precision of Method

The method developed allowed for quantitative determination of the β-naphthylamine produced. Arylamidase activity in soils ranged from 18 to 140 mg β-naphthylamine kg^{-1} of soil h^{-1}. The coefficients of variation of the method described were generally ≤ 4 % (Table 6) and comparable to other methods used for assay of enzymes in soils (Burns, 1978; Tabatabai, 1994). The standard calibration graph is very reproducible. Figure 10 shows calibration curves obtained in January and March 1998 and in September 1999.

Table 6 . Precision of the method

Soil	Arylamidase activity[a]			CV
	Range	Mean	SD	
	— mg β-Napthylamine kg^{-1} soil h^{-1} —			%
Downs	18-20	19	0.61	3.2
Clarion	43-49	46	1.91	4.2
Webster	85-89	87	1.59	1.8
Harps	131-140	136	2.98	2.2

[a] Mean of eight replicated assays; SD = standard deviation; CV = coefficient of variation.

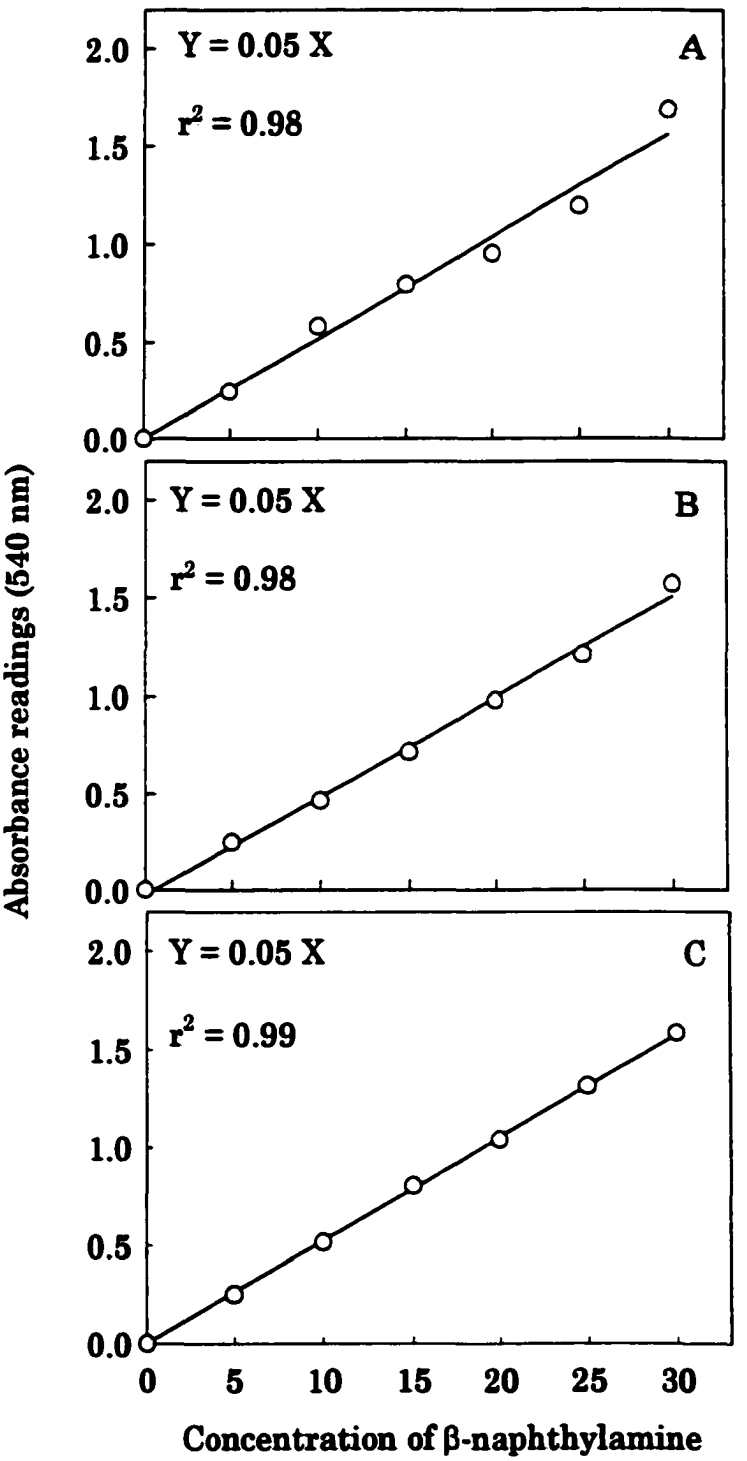


Figure 10. Calibration curve for the β-naphthylamine standards obtained on (A) January 1998, (B) March 1998 and (C) September 1999

PART II. ARYLAMIDASE ACTIVITY OF SOILS: EFFECT OF TRACE ELEMENTS AND RELATIONSHIPS TO ACTIVITIES OF AMIDOHYDROLASES

INTRODUCTION

Trace elements added to soils as impurities in fertilizers, as components of industrial wastes or sewage sludge (Berrow and Webber, 1972; Charter et. al., 1993), or present in fuel oils and gasoline (e.g., Pb, V) (Lagerwerff, 1972; Tyler, 1976) inhibit the activities of soil enzymes. The term *trace element* used here refers to the elements that are, when present in sufficient concentration, toxic to living systems (Page, 1974). The degree of enzyme inhibition varies with the concentration and form of the trace element, the soil investigated, and the type of enzyme assayed (Ladd, 1985; Nannipieri, 1994). Tyler (1981) reported a negative correlation between enzyme activities in soils and their heavy metal content, especially Cu, Pb and Zn. Soil enzymes that have been reported to be inhibited by trace elements are α- and β-glucosidases and α- and β-galactosidases (Tyler, 1974; Eivazi and Tabatabai, 1990), urease (Tyler, 1974; Tabatabai, 1977), phosphatases (Tyler, 1974; Juma and Tabatabai, 1977), arylsulfatase (Al-Khafaji and Tabatabai, 1979), nitrate reductase (Fu and Tabatabai, 1989), L-glutaminase (Frankenberger and Tabatabai, 1991b), L-asparaginase (Frankenberger and Tabatabai, 1991a), L -aspartase (Senwo and Tabatabai, 1999) and cellulase (Deng and Tabatabai, 1995). It is important to understand the relative effect of individual trace elements on the activity of arylamidase (see Part I) because changes in the enzyme activities due to soil

pollution with such compounds might have lasting effects on N cycling and alternately on ecosystem function.

Several intrinsic soil chemical and physical properties (organic C, N, pH, clay and sand) affect the activity of enzymes. Studies with other enzymes of the N cycle (amidohydrolases) have reported they are highly correlated with organic C, total N, and clay content (Tabatabai, 1977; Frankenberger and Tabatabai, 1981, 1991c,d; Senwo and Tabatabai, 1999). The activity of L-asparaginase was significantly correlated to humus, total N, mineral N, and readily hydrolyzed N in non-chernozemic soils (Koloskova and Murtazina, 1978). However, Frankenberger and Tabatabai (1991c) reported a significant correlation between asparaginase activity and organic C and total N, but not with clay content, sand or soil pH of 26 Iowa soils that they studied. The activities of amidase, L-aspartase, L-glutaminase and urease have also been shown to be correlated with organic C, total N and clay content for Iowa soils (Tabatabai, 1977; Frankenberger and Tabatabai, 1981, 1991d; Senwo and Tabatabai, 1999). The same studies have reported the interrelationships among such enzymes involved in the N cycle. Frankenberger and Tabatabai (1991c) reported significant correlations between the activities of L-asparaginase, amidase, and urease in 26 surface soils. Senwo and Tabatabai (1999) reported significant correlations between L-aspartase with L-asparaginase, amidase or urease. None of these studies have been done with arylamidase activity, and there is no information on effect of trace elements on the activity of this enzyme in soils. Therefore, the

objectives of this part were: (i) to evaluate the relative effects of trace elements on arylamidase activity in soils, (ii) to assess the relationship between selected soil properties and arylamidase activity in soils, and (iii) to study the relationship between arylamidase activity and the activities of amidohydrolases (other enzymes involved in N mineralization) in soils.

MATERIALS AND METHODS

Soils

The effects of soil properties on the activity of arylamidase were studied on 26 surface samples (< 2mm, air-dried) selected to obtain a wide range of pH (5.1 to 7.9), organic C (3.6 to 44 g kg^{-1}), total N (0.43 to 3.9 g kg^{-1}) and texture (100 to 356 g kg^{-1} clay and 10 to 640 g kg^{-1} sand) (Table 7). Three air-dried soils and their field-moist counterparts (Monona, Grundy and Downs) were used to study the effect of trace elements on arylamidase activity in soils. Soil pH was determined with a combination glass electrode, organic C by the Mebius method (1960), total N as described by Bremner and Mulvaney (1982), and particle-size distribution by pipette analysis (Kilmer and Alexander, 1949). In the 26 surface soils, the activities of arylamidase (Part I), amidase, L-glutaminase, L-asparaginase (Tabatabai, 1994) and L-aspartase (Senwo and Tabatabai,1996) were assayed.

Trace Elements

The trace elements used were Fisher certified reagent-grade chemicals. Of these, Ag(I), Cd(II), Co(II), Cu(II), Fe(II), Zn(II) and V(IV) were added as the sulfate; Cu(I), Ba(II), Hg(II), Mg (II), Mn(II), Ni(II), Sn(II), Al(III), Cr(III) and Fe(III) were added as the chloride; Pb(II) as the nitrate; and As(III), B(III),

Table 7. Some selected properties of the soils used

Soils		Properties				
Series	Subgroup	pH[a]	Organic C	Total N	Clay	Sand
			g kg^{-1}			
Storden	Typic Eutrochrept	7.8	3.6	0.43	200	414
Ida	Typic Udorthent	7.4	6.0	0.78	160	70
Hayden	Typic Hapludalf	5.8	8.0	0.80	140	530
Hagener	Entic Hapludoll	6.4	9.2	0.93	130	640
Weller	Chromic Aquertic Hapludalf	6.0	12.2	1.40	235	46
Luther	Aeric Ochraqualf	6.4	13.0	1.02	170	330
Gosport	Chromic Oxyaquic Eutrochrept	5.5	23.7	2.04	260	13
Downs	Mollic Halludalf	5.1	14.3	1.38	163	53
Fayette	Typic Hapludalf	7.4	15.0	1.75	179	43
Pershing	Vertic Epiaqualf	6.0	15.7	1.40	291	45
Clinton	Chromic Verric Hapludalf	6.0	16.5	1.63	312	19
Edina	Vertic Argialboll	6.2	19.5	1.78	250	10
Tama	Typic Argiudoll	7.5	20.4	1.97	290	30
Marshall	Typic Hapludoll	6.0	20.6	1.96	280	10
Shelby	Typic Argiudoll	6.7	24.6	2.31	260	330
Monona	Typic Hapludoll	5.7	25.4	2.54	210	58

Sharsburg	Typic Argiudoll	6.0	25.4	2.29	330	20
Muscatine	Aquic Hapludoll	7.3	26.0	2.33	300	30
Grundy	Aquertic Argiudoll	6.1	26.9	1.90	248	48
Ames	Typic Albaqualf	6.7	29.9	2.29	100	570
Clarion	Typic Hapludoll	6.3	31.2	2.35	264	250
Webster	Typic Endoaquoll	6.9	32.4	3.80	280	302
Nicollet	Aquic Hapludoll	7.0	33.8	2.19	254	316
Canisteo	Typic Endoaquoll	7.4	35.2	3.02	312	190
Okoboji	Cumulic Vertic Endoaquoll	7.0	43.0	3.90	261	304
Harps	Typic Calciaquoll	7.9	44.0	2.43	356	188

[a]Soil: water ratio (1:2.5).

Se(IV), As(V), Mo(VI), W(VI) and Ti(IV) were added as $NaAsO_2$, $Na_2B_4O_7$, H_2SeO_3, Na_2HAsO_4, Na_2MoO_4, Na_2WO_4 and $TiOSO_4$, respectively.

Assay of Arylamidase Activity

The effect of trace elements on arylamidase activity was studied by treating 1-g of soil in a 25-mL Erlenmeyer flask with 1 mL of solution containing 5 μmol of the trace element. The solution was added dropwise to moisten the whole soil sample. After 30 min of equilibration, the sample was treated with 2 mL of 0.1 *M* THAM buffer (pH 8.0) and 1 mL of 8.0 m*M* L-leucine β-naphthylamide hydrochloride. Because the soil sample was treated with 1 mL of trace-element solution, 2 mL of the buffer was used instead of the 3 mL recommended. The amount of β-naphthylamine produced was determined as described in Part I. The arylamidase activity values obtained for the soils treated with trace elements were compared with that obtained with a 1-g soil sample amended with 1 mL of deionized water instead of the trace element. The percentage of inhibition by each trace element was calculated from [(A – B)/A] 100, where A is the arylamidase activity of non-treated soil and B is the arylamidase activity of the trace element treated soil. Results reported are averages of duplicate assays, expressed on a moisture-free basis. Moisture was determined from weight loss after drying soil at 105°C for 48 h. At all data points reported in the figures, the differences between the duplicate values were smaller than the point size.

RESULTS AND DISCUSSION

Effect of Trace Elements

The degree of inhibition or activation of arylamidase activity by each of the 25 trace elements varied among the soils and their conditions (air-dried or field-moist), without a definite trend (Tables 8 and 9). Because the coefficient of variation values of the assay method used were < 4% (see Part I), the percentages of inhibition or activation values that are < 4% are not significantly different from the activity values obtained when the soil was treated with 1 mL of water instead of the trace element solution. The least inhibition (3 %) was observed with Fe (II) in the air-dried Grundy soil, Ba (II) in the air-dried Downs soil and Ti (IV) in the in field-moist Monona soil. Other elements that showed relatively low inhibition were: Se (IV) (4-37%) and Mo (VI) (7-9%). The inhibition values with Cu (I) ranged from 13 to 52%, and those with Cu (II) ranged from 35 to 53%. The trace elements Ag (I), Hg (II) and Cd (II) were the most effective inhibitors (55- 90%) of arylamidase activity in both air-dried and field-moist soils. Hg (II) has also been reported to have a strong inhibitory effect on the activities of amidohydrolases, suggesting that all these enzymes, as well as arylamidase, contain sulfhydryl groups in their active sites (Frankenberger and Tabatabai, 1981, 1991c,d; Senwo and Tabatabai, 1999).

The trace elements Co (II), Mg (II), Mn (II), B (III), and As (V) activated this enzyme in both air-dried and field-moist soils (Table 9). As (III) inhibited

Table 8. Inhibition of arylamidase activity in soils by trace elements

Trace element		Inhibition of arylamidase activity (%) in soils specified[a]					
Element	Oxidation state	Monona		Grundy		Downs	
		AD	FM	AD	FM	AD	FM
Ag	I	75	64	84	64	87	80
Cu		25	14	19	21	13	52
Ba	II	15	13	4	16	3	26
Cd		81	82	55	66	78	64
Co		·	·	·	·	·	·
Cu		35	41	38	49	41	53
Fe		4	21	3	22	14	22
Hg		69	72	81	82	85	90
Mg		·	·	·	·	·	·
Mn		·	·	·	·	·	·
Ni		30	28	32	23	37	20
Pb		17	22	24	40	25	24
Sn		32	19	26	21	30	37
Zn		28	24	27	52	26	50
Al	III	17	7	6	24	8	40
As		6	16	10	5	12	29
B		·	·	·	·	·	·
Cr		15	17	14	31	32	26
Fe		25	28	25	15	29	52
Se	IV	7	12	4	42	30	27
Ti		18	3	7	40	44	46
V		18	11	10	23	19	52
As	V	·	·	·	·	·	·
W	VI	·	12	·	18	·	46
Mo		·	7	·	7	·	9
LSD[b] ($P < 0.05$)		6	7	8	8	7	10

[a] Arylamidase activity of non-treated soils (mg β-naphthylamine kg^{-1} soil h^{-1}); AD = air dried (Monona = 62 ; Grundy = 43 ; Downs = 11); FM = field moist (Monona = 76; Grundy 41; Downs = 14). The sign - indicates activation, see Table 9.

[b] LSD = least significant difference.

Table 9. Activation of arylamidase activity in soils by trace elements

Trace element		Activation of arylamidase activity (%) in soils specified[a]					
Element	Oxidation state	Monona		Grundy		Downs	
		AD	FM	AD	FM	AD	FM
Co	II	26	33	17	7	37	28
Mg		1	8	13	2	14	24
Mn		5	16	19	15	6	49
B	III	21	3	11	8	5	23
As	V	19	5	34	13	14	33
W	VI	16	-	21	-	13	-
Mo		4	-	4	-	5	-
LSD[b] ($P < 0.05$)		5	7	5	6	9	11

[a] Arylamidase activity of non-treated soils (mg β-naphthylamine kg^{-1} soil h^{-1}); AD = air dried (Monona = 62 ; Grundy = 43 ; Downs = 11); FM = field moist (Monona = 76; Grundy = 41; Downs = 14). The sign $^{-}$, indicates inhibition from Table 8.

[b] LSD = least significant difference.

but As (V) activated this enzyme in soils. The elements W (VI) and Mo (VI) activated this enzyme in the air-dried soils (4-21%), but acted as inhibitors in the field-moist soils (7-46%) (Tables 8 and 9). This difference on the effect of trace elements on arylamidase activity of air-dried and field-moist soils suggest that air-drying may have resulted in more exposure of the enzyme to the trace elements upon rewetting; air-dried soil aggregates break down upon rewetting (Wiersum, 1962). The variation in the effect of trace elements on the activity of arylamidase in a soil at different conditions (field moist and air-dried) and among soils may also be related to the different soil-trace element interactions despite that the activity of the enzyme was assayed after 30 min of equilibration of the soil-trace element solution mixture. It is possible that because of the variation in the original soil pH values before addition of THAM buffer for assay of the enzyme activity, the ionic species of the trace elements varied among the soils. Furthermore, there are other trace-element reaction processes in soils, e.g., cation-exchange, reaction with functional groups of organic matter, adsorption and precipitation reactions that could consume significant portions of the added trace elements. These processes are perhaps responsible for the variation in the inhibition or activation observed among the soils used (Lindsay, 1979; Karmarkar and Tabatabai, 1993).

Studies with pure preparations of arylamidase from rat brain have reported that treatment with ethylenediamine tetraacetate resulted in loss of activity, which could be restored by the addition of 0.1 mM of Zn (II), Co (II), Mn

(II), Ca (II), Mg (II) or La (III) (Marks et al., 1968). However, Gary and Santiago (1977) reported that further addition of 1-2 mM, Mg (II), Mn (II), Co (II), or Zn (II) inhibited the activity slightly (10-30%). The activation of arylamidase activity in soils by Co (II) is in agreement with that reported by Marks et al. (1968) and Hiwada et al. (1980) for purified enzyme preparations. The activation by Mg (II) and Mn (II) was also reported by Appel (1974).

Control studies with 5 μmol of NaCl and K_2SO_4 g^{-1} soil indicated that K^+, Na^+, Cl^-, and SO_4^{2-} associated with the salts of the trace element studied did not affect arylamidase activity in soils. Also, NO_3^- did not affect the activity of this enzyme in soils.

The pH of the trace element solution varied considerably, ranging from 2.1 for $FeCl_3 \cdot 6H_2O$ solution to 10.2 for the $NaAsO_2$ solution. However, the use of THAM buffer ensured a constant pH of 8.0 ± 0.2 in the reaction mixture. Therefore the observed inhibition or activation of arylamidase in soils in the presence of the trace elements studied was not a result of changes in pH of the reaction mixture but of the reaction between the free trace element in solution and the functional groups of arylamidase.

Effect of Soil Properties

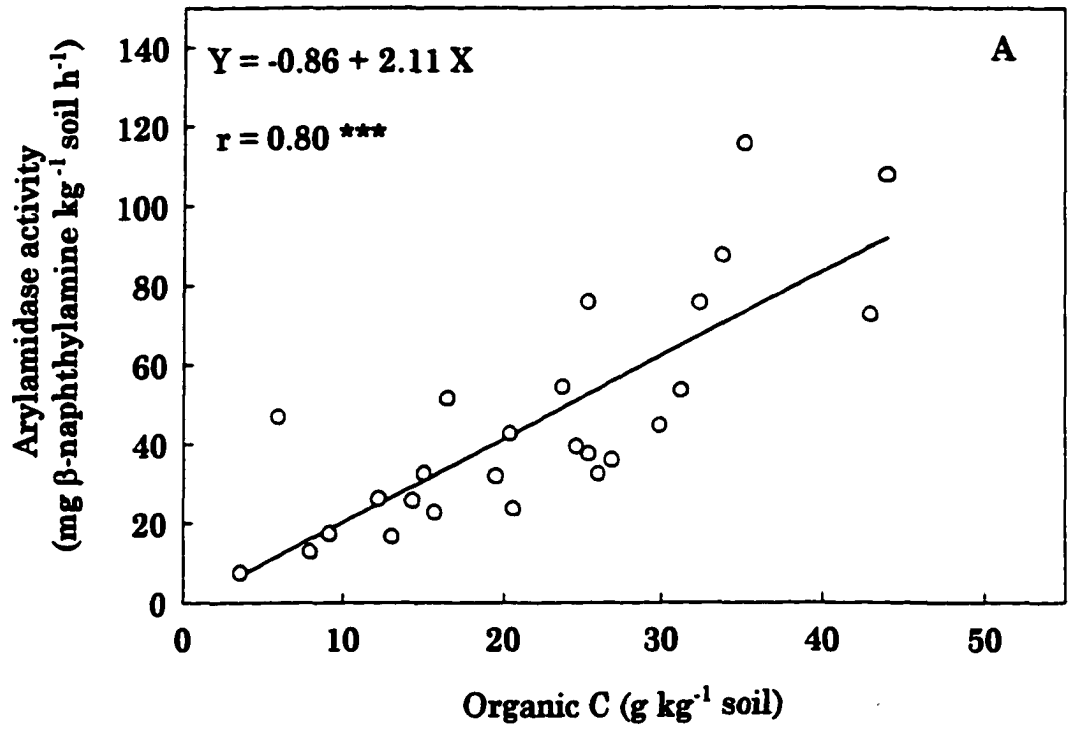
Several soil properties (i.e., contents of organic C, total N, clay or sand, soil pH, etc.) affect the activity of soil enzymes, and the interrelationships between these properties that exist in soils affect differently the amount of

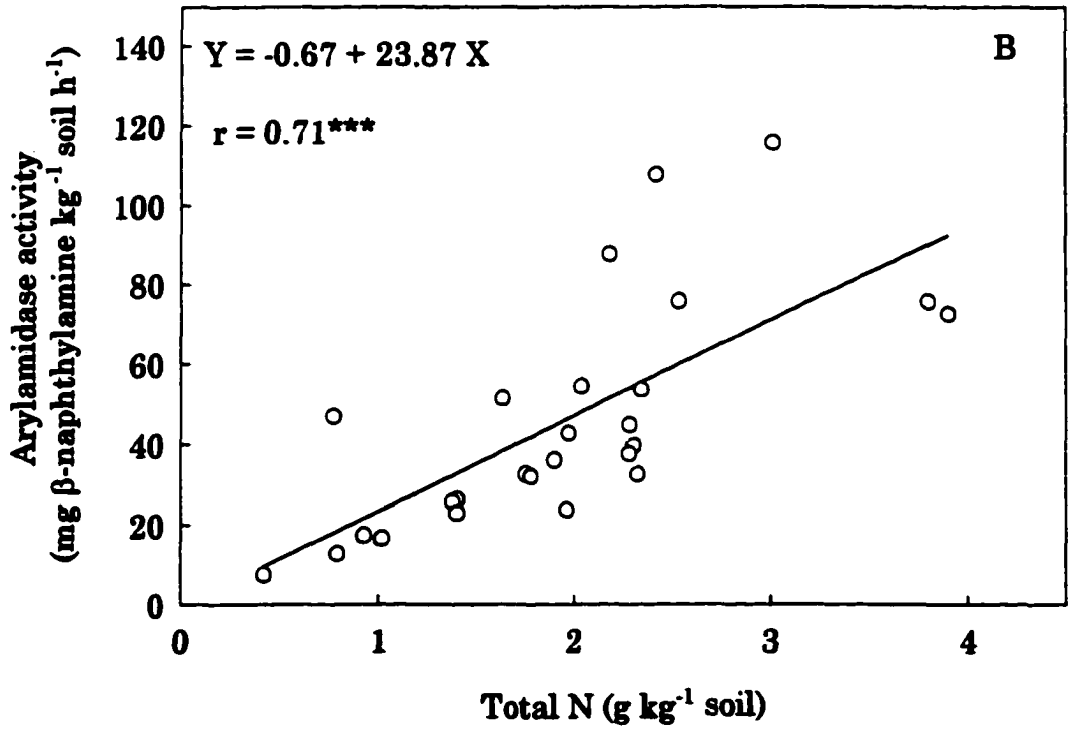
enzyme activities observed. Many researchers have found the content of organic C in soil is correlated with enzyme activity (Tabatabai and Bremner, 1970; Speir, 1977; Frankenberger and Dick, 1983; Frankenberger and Tabatabai, 1981, 1991c,d; Senwo and Tabatabai, 1999). In this study, a significant correlation ($r = 0.80^{***}$) was found between the activity of arylamidase and the content of organic C in the 26 soils studied (Figure 11). The correlation (r value) found in this study was very close to the reported value for the amidohydrolases including amidase (0.74***, Frankenberger and Tabatabai, 1981), L-glutaminase (0.79***, Frankenberger and Tabatabai, 1991d), L-asparaginase (0.86***, Frankenberger and Tabatabai, 1991c), urease (0.80***, Tabatabai, 1977), and L-aspartase (0.85***, Senwo and Tabatabai, 1999).

The activity of arylamidase was also significantly correlated with the content of total N ($r = 0.71^{***}$) and with clay ($r = 0.49^{**}$) (Figures 11B and 12), but not with the content of sand or the pH of the soils examined. Senwo and Tabatabai (1999) found similar r values for the relationship between the activity of aspartase and soil total N (0.73***) and clay content (0.44*). A significant correlation between enzyme activities and clay content in soils is expected because the persistence and stability of the soil enzymes are highly related to the binding to clay-organic matter complexes (McLaren, 1975; McLaren et al., 1975).

The lack of significant correlation between arylamidase activity and soil pH is not surprising, because the 26 surface soils tested varied in other chemical

Figure 11. Relationships between arylamidase activity and the contents of organic C (A) and total N (B) in soils. * indicates $P < 0.001$**


Y = -0.86 + 2.11 X
r = 0.80 ***
A
Arylamidase activity
(mg β-naphthylamine kg-1 soil h-1)
Organic C (g kg-1 soil)
0
20
40
60
80
100
120
140
0
10
20
30
40
50


Y = -0.67 + 23.87 X
r = 0.71***
B
Arylamidase activity
(mg β-naphthylamine kg-1 soil h-1)
Total N (g kg-1 soil)
0
20
40
60
80
100
120
140
0
1
2
3
4

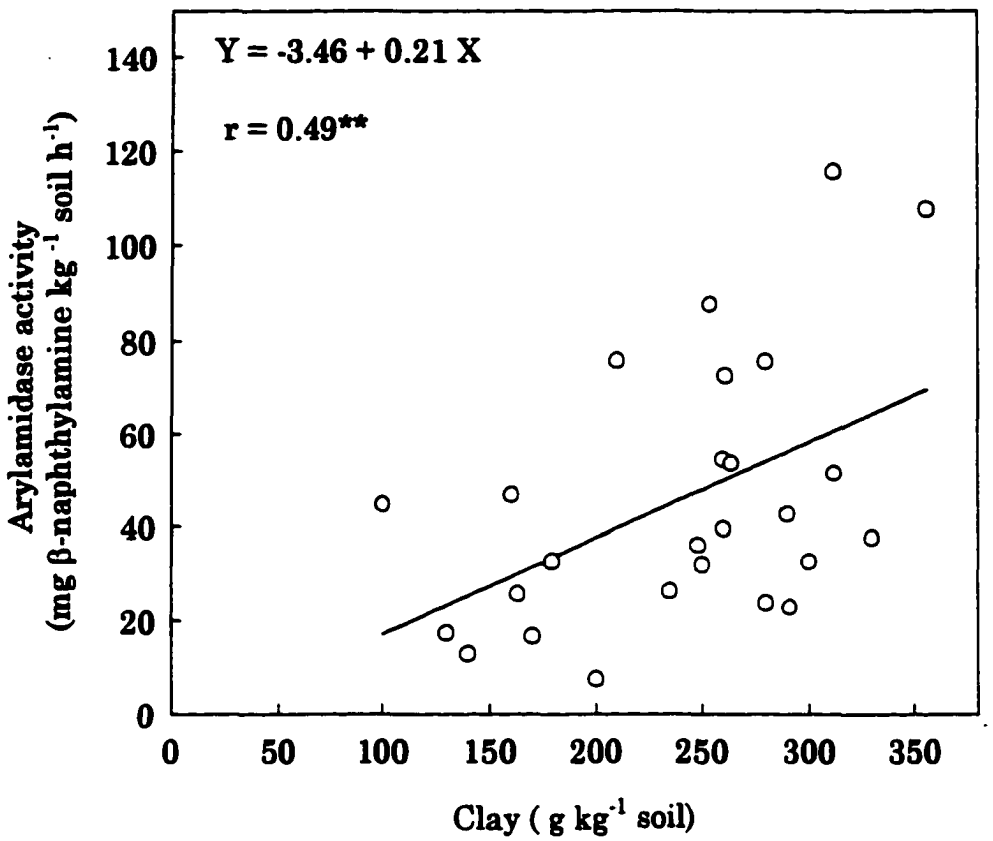


Figure 12. Relationship between the activity of arylamidase and the content of clay in soils. ** indicates $P < 0.05$

properties. It is well known that variation in organic matter content among soils significantly alters enzyme activities, as demonstrated in this work (Figure 11A). However, recent studies showed that arylamidase activity was significantly correlated ($r = 0.74^{***}$) with soil pH of one acid agricultural soil limed under field conditions to pH values ranging from 4.9 to 6.9 (see Part V).

Relationship between Arylamidase Activity and Activities of Amidohydrolases

Amidohydrolases are important in the mineralization of organic N in soil, and consequently these enzymes were assayed to determine whether there was a relationship between the activity of each of these enzymes and arylamidase activity in soils. The activity of arylamidase was significantly correlated with the activities of L-asparaginase ($r = 0.91^{***}$), L-aspartase ($r = 0.90^{***}$), urease ($r = 0.87^{***}$), L-glutaminase ($r = 0.84^{***}$) and with amidase ($r = 0.39^{*}$) in the 26 surface soils studied (Figure 13). The significant correlation between the arylamidase activity and the activities of the amidohydrolases suggests that these enzymes have similar origin, location or persistence in soils. Senwo and Tabatabai (1999) also reported similar relationships between the activities of some amidohydrolases and L-aspartase, and a less significant correlation between L-aspartase and amidase ($r = 0.44^{*}$). Other studies have found more significant correlations between the activity of amidase and other amidrohydrolases such as L-asparaginase ($r = 0.82^{***}$), L-glutaminase ($r = 0.82^{***}$) or urease ($r = 0.73^{***}$) (Frankenberger and Tabatabai, 1981; 1991 c,d).

Figure 13. Relationships between arylamidase activity and the activities of L-asparaginase, L-aspartase, L-glutaminase and urease in soils. * indicates $P < 0.001$**

Y = 0.46 + 0.80 X
r = 0.91***
L-Asparaginase activity
mg NH4+-N produced kg-1 soil 2h-1
Y = 0.23 + 4.2 X
r = 0.90 ***
L-Aspartase activity
mg NH4+-N produced kg-1 soil 24h-1
Y = 55.2 + 6.2 X
r = 0.84***
L-Glutaminase activity
mg NH4+-N produced kg-1 soil 2h-1
Y = -7.2 + 2.4 X
r = 0.87 ***
Urease activity
mg NH4+-N produced kg-1 soil 2h-1
Arylamidase activity (mg β-Naphthylamine kg-1 soil h-1)

PART III. EFFECT OF TOLUENE ON ARYLAMIDASE ACTIVITY IN SOILS

INTRODUCTION

Recently, a method was developed for the assay of arylamidase activity in soils (Part I). Preliminary experiments demonstrated that the activity of this enzyme is inhibited by toluene. Toluene is often used in enzyme assays because as an antiseptic agent it has the ability to inhibit the synthesis of soil enzymes by living cells, and as a plasmolytic agent it has the ability to remove the lipid components of the microbial cell membrane that enables the release of enzymes and/or the diffusion of substrates and products across the cell membrane (Skujins, 1967). Frankenberger and Johanson (1986) reported that toluene enhanced by 1.30 to 1.34-fold the activities of the soil enzymes arylsulfatase and urease suggesting that its plasmolytic character was affecting the intracellular enzyme pool of the measured activities. Work on assay methods of many soil enzymes, including urease, L-asparaginase, L-glutaminase, L-aspartase, amidase, α or β-glucosidases, α or β-galactosidases, arylsulfatase, acid and alkaline phosphatases and phosphodiesterase, showed that the addition of 5 to 10 % of toluene is sufficient to increase the enzyme activities due to supression of the microbial proliferation (Tabatabai, 1994; Senwo and Tabatabai, 1996).

Because toluene inhibited arylamidase activity in soils, it became necessary to identify the type of inhibition involved. Each type of inhibition affects differently the enzyme-substrate (ES) complex. In competitive inhibition, the inhibitor (I) competes with the substrate (S) for binding to the active site of

the enzyme. This causes alteration of the affinity of the enzyme for the substrate (K_m) because it interferes with the formation of the substrate-enzyme (ES) complex by forming enzyme-inhibitor complex (EI). In non-competitive inhibition, the inhibitor does not compete with the substrate, because it does not bind to the active site of the enzyme. This allows the ES complex formation, but with a lower reaction velocity (V_{max}) and, thus, it does not change the K_m value. The mixed type inhibition is a special type of non-competitive inhibition in which the inhibitor binds to both: the enzyme and the enzyme-substrate complex.

Knowledge of the kinetic parameters of soil enzymes, in this case, arylamidase, is used to characterize enzyme reactions and to predict the reaction rate and substrate concentration needed under certain conditions. For example, information about the activation energy (E_a), enthalpy of activation (ΔH_a) and Q_{10} values are used for predicting the efficiency of enzyme-catalyzed reactions, and to calculate the reaction rates at some desired temperatures. The Michaelis-Menten constant (K_m) is one of the fundamental constants in enzyme chemistry because it is an indication of the relative affinity of an enzyme for the substrate. The K_m value is independent of the enzyme concentration and is valuable in studies of enzyme kinetics because it describes the substrate concentration at which the reaction velocity is half maximal (V_{max}).

Because of the lack of information on the activity of arylamidase in soils, and toluene is commonly used in assay of soil enzymes, and because of the importance of this enzyme in a rate-limiting step of the hydrolysis of organic N

in soils, this work was carried out: (i) to compare the kinetic and thermodynamic parameters of the reaction catalyzed by arylamidase in soils in the presence and absence of toluene, and (ii) to assess the type of inhibition exerted by toluene on the arylamidase protein in soils.

MATERIAL AND METHODS

Soils and their Properties

The soils used in this study are reported in Table 10. The methods used to characterize the soils are those reported in Part I.

Reagents

The preparation of the reagents required for assay of arylamidase activity in soils are described in Part I.

Toluene--Fisher certified reagent.

Experiments

This study involved similar experiments as those done in Part I for determining the optimal conditions of assay the activity of arylamidase in soils in absence of toluene, but in this case by using toluene. The experiments were: the effect of toluene on activity of this enzyme as described by optimal pH, substrate concentration, amount of soil, time of incubation, temperature of incubation, air-drying of field-moist soils, and preheating temperature, and selected inhibitors. The results obtained in the experiment of the optimal substrate concentration for arylamidase activity in soils were used to calculate and compare the kinetics parameters K_m and V_{max} values with and without toluene. These values were calculated using the three possible linear

Table 10. Selected properties of the soils used

Soil		pH[a]					
Series	Subgroup	H_2O	$CaCl_2$	Org. C	Total N	Clay	Sand
				g kg^{-1}			
Downs	Mollic Hapludalf	5.1	4.3	14.3	1.38	163	53
Grundy	Aquic Argiudoll	6.3	5.2	15.4	1.09	300	11
Monona	Typic Hapludoll	5.7	5.1	25.4	2.54	210	58
Webster	Typic Haplaquoll	6.9	6.5	32.4	3.80	280	302

[a]Soil:water or soil: 0.01 *M* $CaCl_2$ ratio, 1:2.5.

transformations of the Michaelis-Menten equation; Lineweaver-Burk plot, Eadie-Hofstee plot, and Hanes-Woolf plot. The results obtained from the plots were used to determine the type of inhibition exerted by toluene on the activity of arylamidase. The thermodynamic parameters in the presence and absence of toluene; i.e., energy of activation (E_a), enthalpy of activation (ΔH_a) and temperature coefficients (Q_{10}) were calculated from the arylamidase activity values obtained at different incubation temperatures.

Assay of Arylamidase Activity

The procedure to assay the activity of arylamidase is described in Part I. The only modification to this procedure was the addition of 0.1 mL of toluene to the 1-g soil sample (air-dried, <2mm) in the 25-mL Erlenmeyer flask. After mixing the soil-toluene mixture, the sample was treated with 3 mL of 0.1 *M* THAM buffer (pH 8.0) and 1 mL of 8.0 *mM* L-leucine β-naphthylamide hydrochloride and the same procedure described in the Material and Methods section of Part I was followed.

Controls were also included as described for the assay, but the 1 mL of the substrate was added after incubation. Subsets of samples were used to study the factors affecting the activity of this enzyme in presence and absence of toluene. These were selected to give ranges in the activity values and to avoid overlapping of the curves of enzyme activity obtained. All results reported are averages of duplicate assays on air-dried soils, and are expressed on a moisture-

free basis. Moisture was determined from the loss in weight after drying at 105°C for 36 h. At all data points reported in the figures, the differences between the duplicate values were smaller than the point size.

RESULTS AND DISCUSSION

Toluene is added to soil suspension to increase the activity of the enzymes under the assay conditions, but previous experiments with arylamidase showed that the addition of toluene decreased the activity of this enzyme. Thus, in this work, to understand the biochemistry of arylamidase and the effect of toluene on the activity of the enzyme, the various kinetic parameters of the reaction catalyzed by arylamidase in soils were studied. The soil samples were incubated in presence and absence of toluene following the method described in Part I for assay of the activity of arylamidase. Results will be reported using the same subheadings as in Part I.

Buffer pH

The experiment to ascertain the optimal pH for arylamidase activity in soils using 2 m*M* of L-leucine β-naphthylamide in the presence of THAM buffer at pH values ranging from 5 to 10 done in Part I was repeated but in presence of toluene. Results showed that the rate of β-naphthylamine produced was always optimal at buffer pH = 8.0 in presence and absence of toluene for the Webster, Grundy and Downs soils (Figure 14). However, with all the soils used and the different pH of the buffer tested, the activity of arylamidase was greater in the absence than in the presence of toluene. For example, at optimal pH value, the activity value of arylamidase of the Webster soil was 54 mg β-napthylamine kg^{-1} soil h^{-1} in presence of toluene compared with 86 mg β-napthylamine kg^{-1} soil h^{-1}

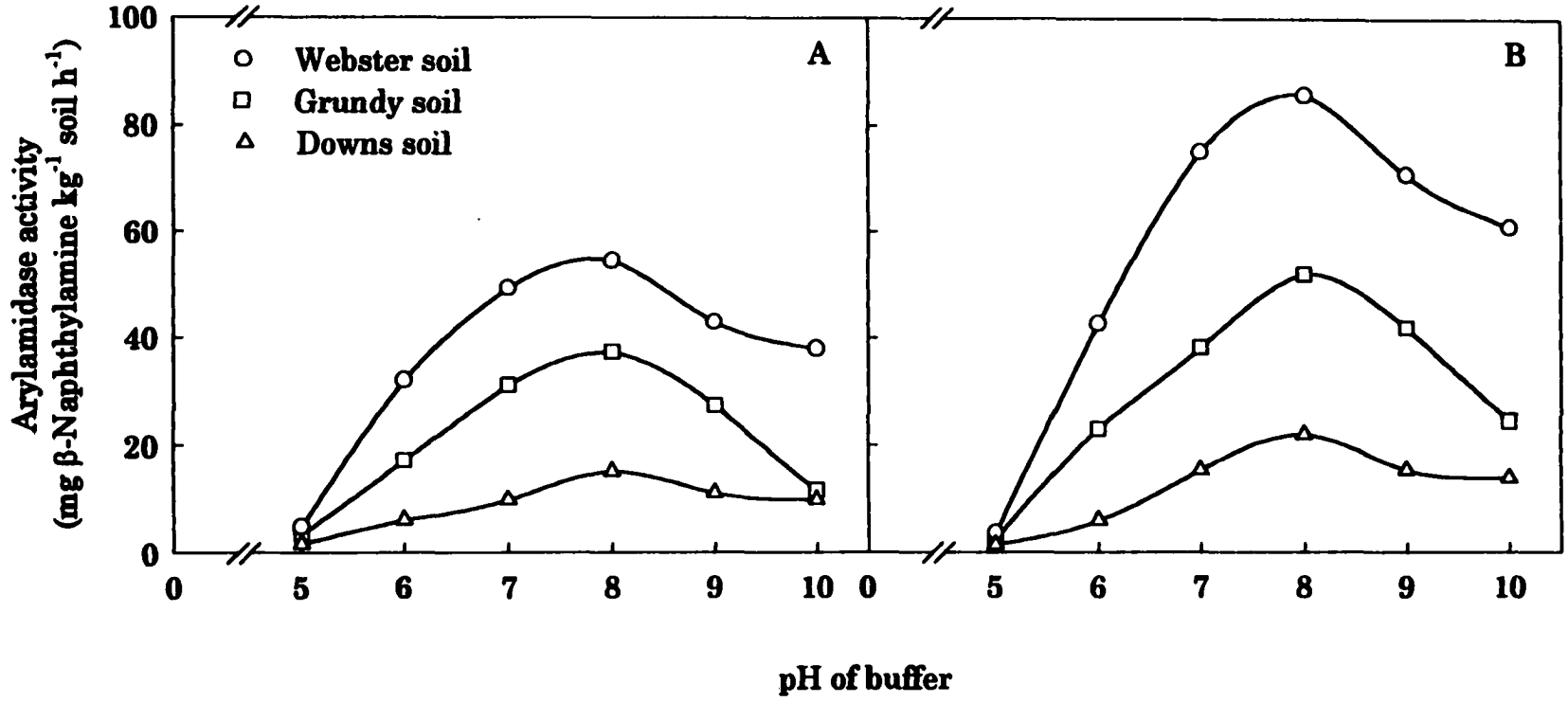


Figure 14. Effect of pH of buffer on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) No-toluene

in the absence of toluene. The lower values of activity observed in the presence of toluene were indicative of an inhibitory effect of toluene on the activity of this enzyme.

Substrate Concentration and Amount of Soil

The initial rates of arylamidase activity in soils were measured at various substrate concentrations in order to determine the effect of toluene on the optimal substrate concentration for this enzyme (K_m value). The form of the Michaelis-Menten equation is such that approximately 10 to 90 % of V_{max} are achieved at substrate concentrations corresponding to $K_m \times 10^{-1}$ and $K_m \times 10$, respectively. Figure 15 shows that, with and without toluene, the reaction velocity was increased with increases in the substrate concentration, and again showed that the concentration (2 m*M*) adopted was satisfactory for the assay of arylamidase activity in soils because the reaction essentially followed zero-order kinetics. However, the addition of toluene decreased the maximum reaction velocity (V_{max}) of this enzyme and, thus, it affected the K_m value (substrate concentration at ½ V_{max}). The results shown in this plot also demonstrate that even though the activity of arylamidase was greater in the absence of toluene, the rate of the reaction at this substrate concentration was still dependent on the enzyme concentration in the 1 g of soil used.

The linear relationship between the amount of soil and the amount of β-naphthylamine produced also showed that 1 g of soil was satisfactory for

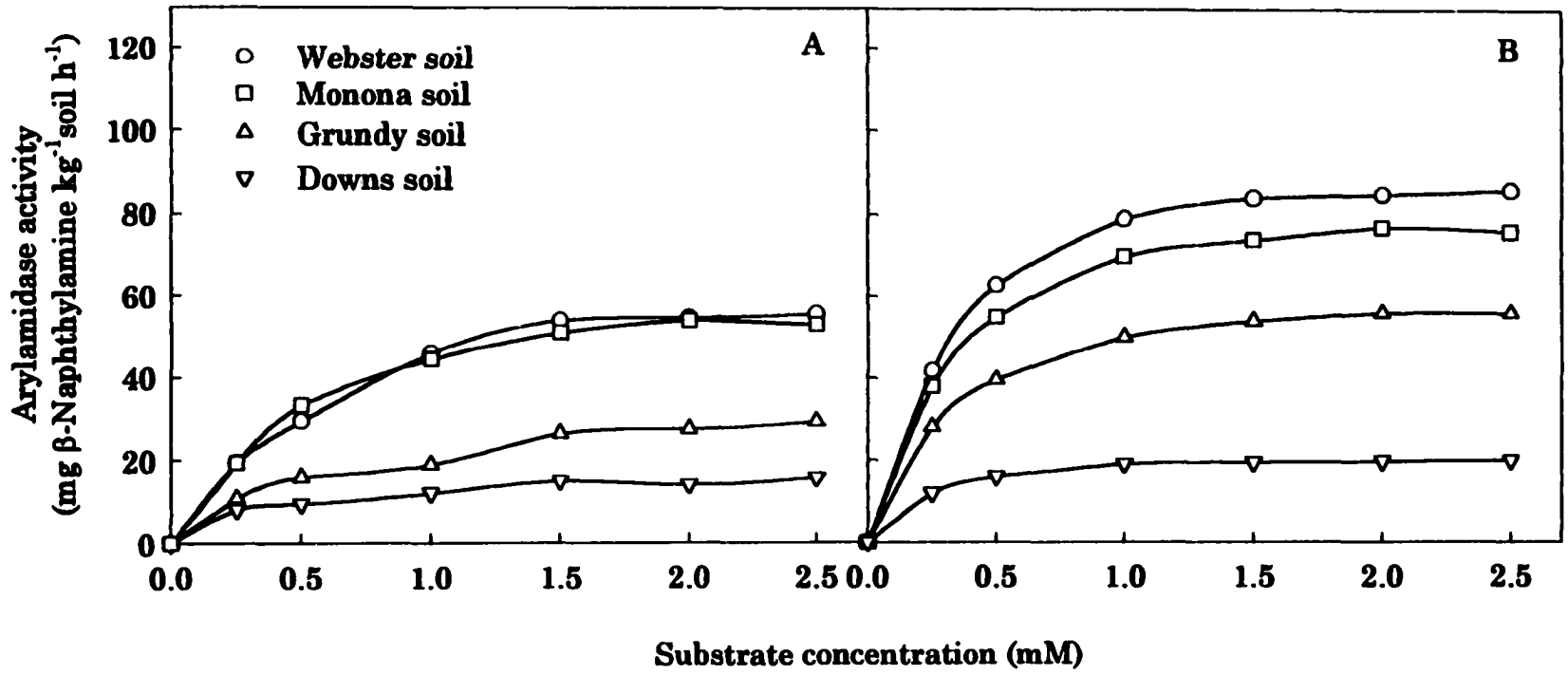


Figure 15. Effect of substrate concentration on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) No-toluene

assaying the activity of this enzyme with or without toluene (Figure 16). Results (linearity of the curves with 1g of soil) demonstrate that even though higher values of activity values were obtained in the absence of toluene, the procedure described (1 h of incubation) measures arylamidase activity without complication from microbial proliferation.

Time and Temperature of Incubation

Experiments to test the effect of incubation time on the activity of an enzyme are important because complications such as microbial growth or the assimilation of enzymatic products by microorganisms may occur. However, results reported in Figure 17 show that the relationship between the amount of product formed and the time of incubation was linear (zero-order reaction) with and without toluene, demonstrating that, as stated in Part I, the enzyme is stable and retains it full activity, and that the possible complications from microbial proliferation mentioned above did not occur. This experiment showed that the incubation time of 1 h, reported for the assay in Part I, allowed ample time for accumulation of β-naphthylamine even when samples are incubated without toluene.

Results showed that, with and without toluene, all soils tested showed faster rates with increasing temperature up to a temperature of 60°C above which the enzyme activity decreased due to enzyme denaturation (Figure 18). Even though the optimal activity of arylamidase was 60°C, the temperature of

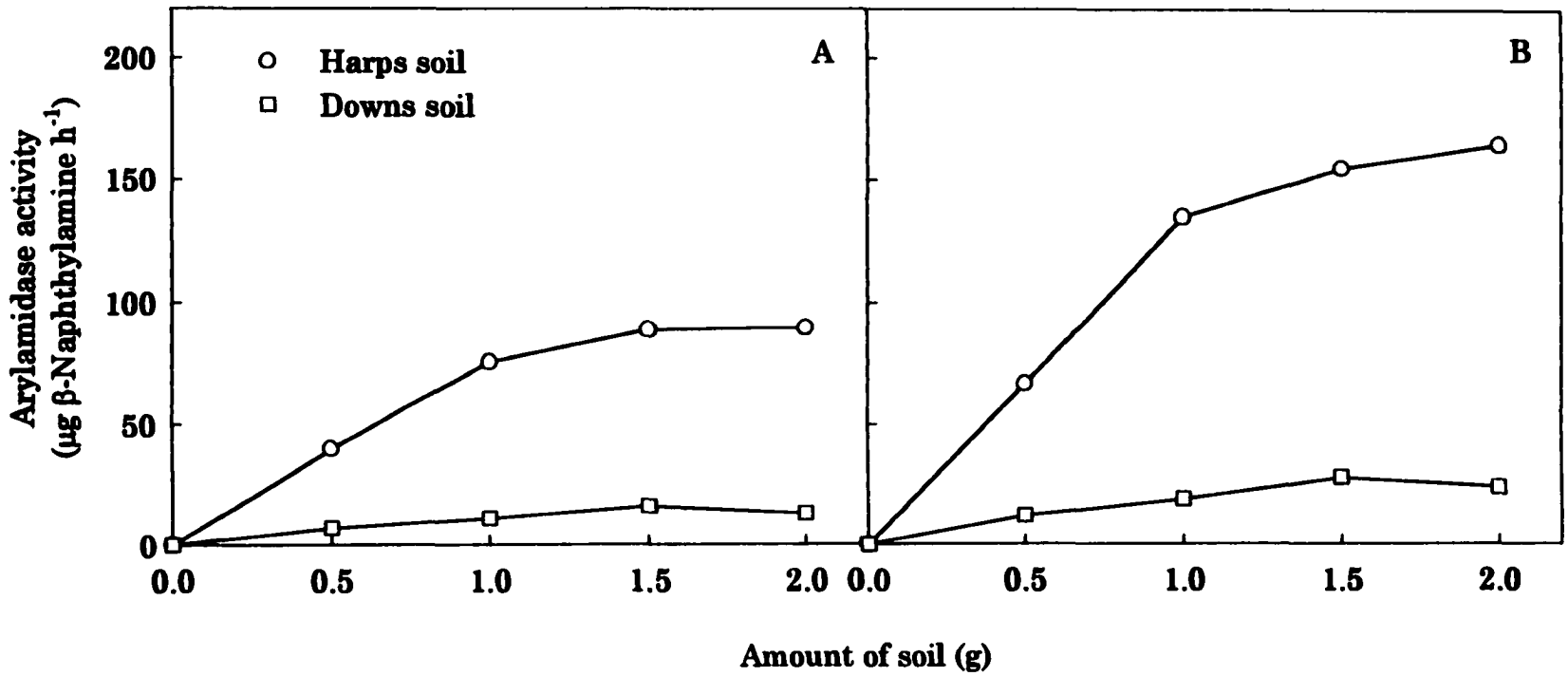


Figure 16. Effect of amount of soil on production of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) No-toluene

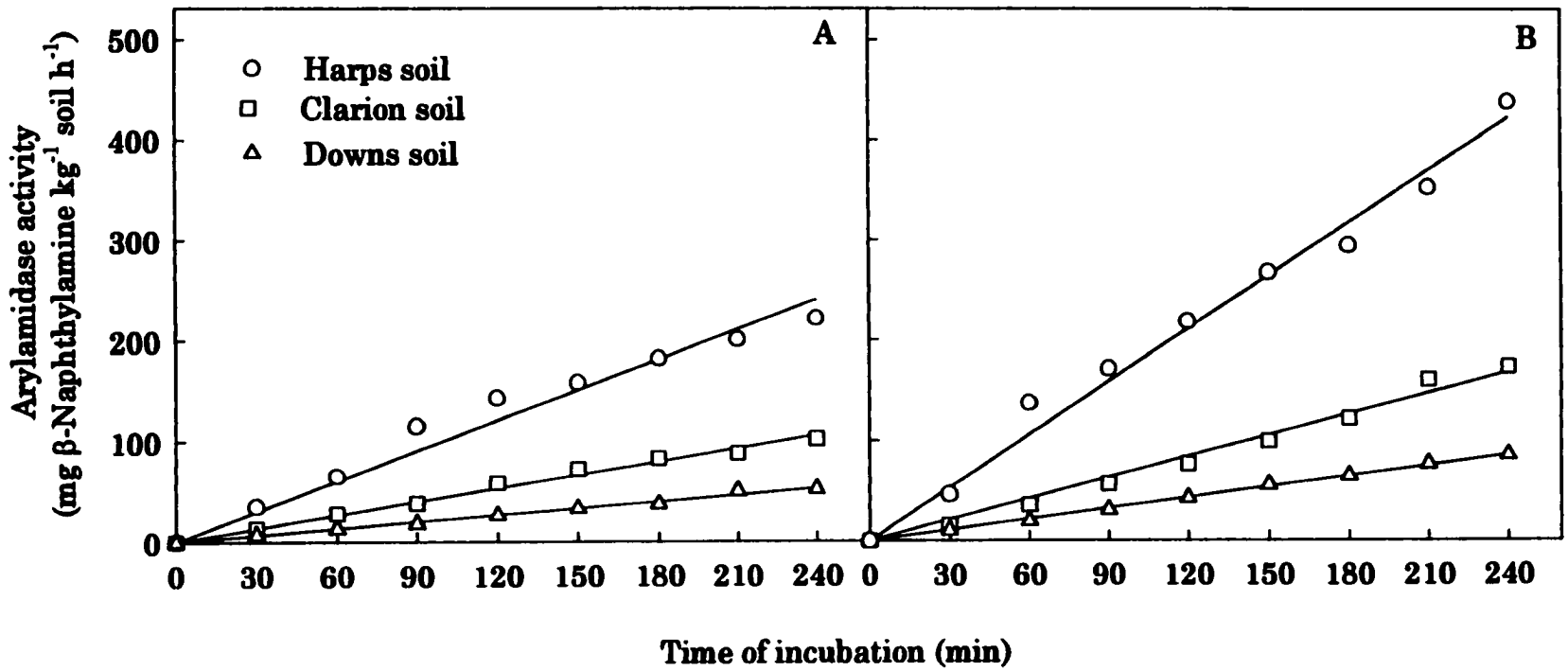


Figure 17. Effect of time of incubation on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) No-toluene

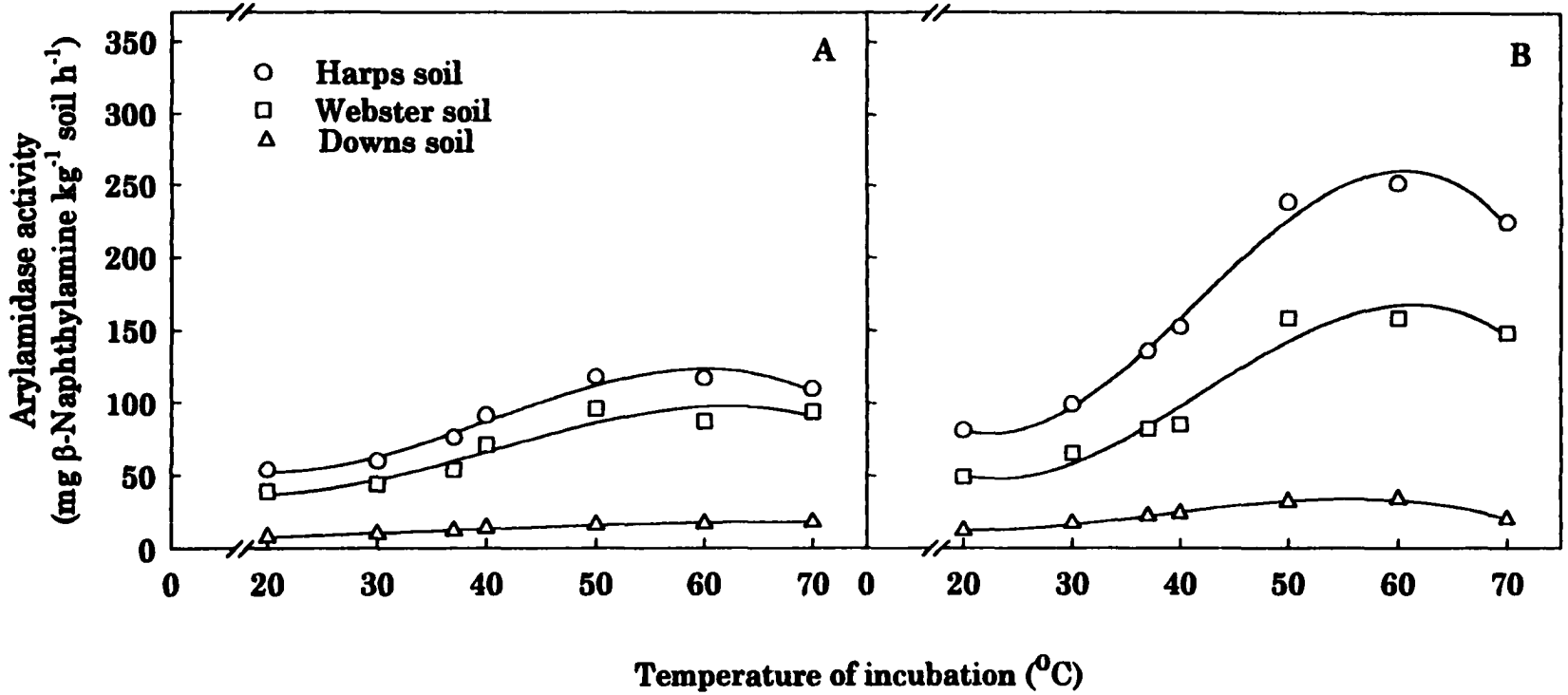


Figure 18. Effect of incubation temperature on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) no-toluene

the assay procedure can be 37°C, as it has been extensively used for other enzymes in many biological materials, including soils (Tabatabai, 1994).

The temperature used in drying field-moist soils and storing air-dried samples affect the enzyme activities (Tabatabai, 1994) and thus, the effect of preheating temperature on the stability of arylamidase in air-dried soils with and without toluene was studied for this enzyme. Soil samples were exposed to temperatures ranging from 20 to 120°C for 2 h, and the arylamidase activity was assayed at 37°C. Incubation of the air-dried soils in presence or absence of toluene showed that the activity of this enzyme was stable up to 60°C (Figure 19). Results reported in Part I showed that, without toluene, the air-dried soils contained residual activity after preheating at 120°C for 2 h compared with its field-moist counterparts, and again residual activity was still observed with the addition of toluene.

Activation Energy, Enthalpy of Activation, and Kinetic Parameters

Temperature dependence of enzyme-catalyzed reactions is well documented. The dependence of the rate constant on temperature (below the inactivation temperature) of an enzyme-catalyzed reaction can be represented by the Arrhenius equation. The Arrhenius equation plot for arylamidase activity in the soils studied was linear between 20 and 50°C in presence and absence of toluene (Figure 20). The activation energies of the enzyme reaction in the soils were obtained from the slope, and the values for the samples incubated with and

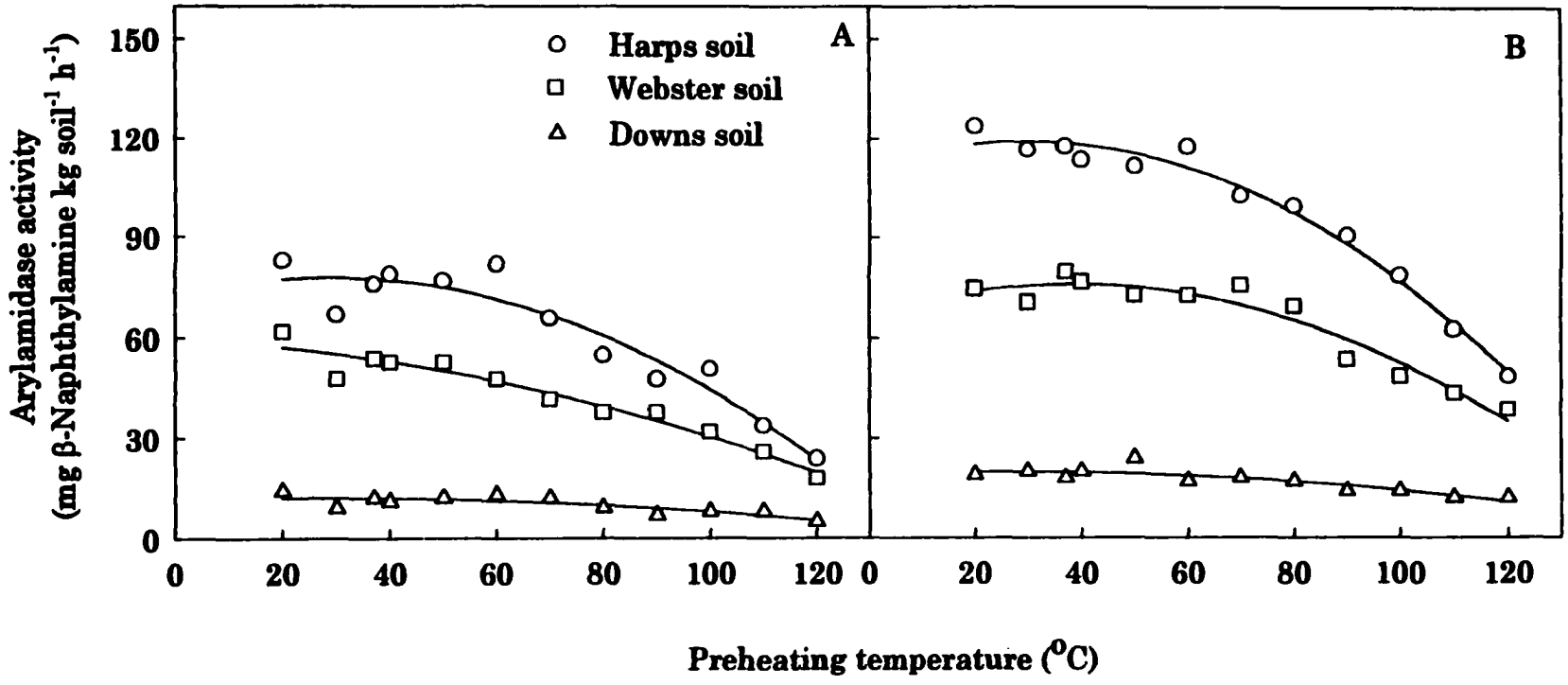


Figure 19. Effect of preheating temperature on release of β-naphthylamine in assay of arylamidase activity in soils. (A) Toluene; (B) no-toluene

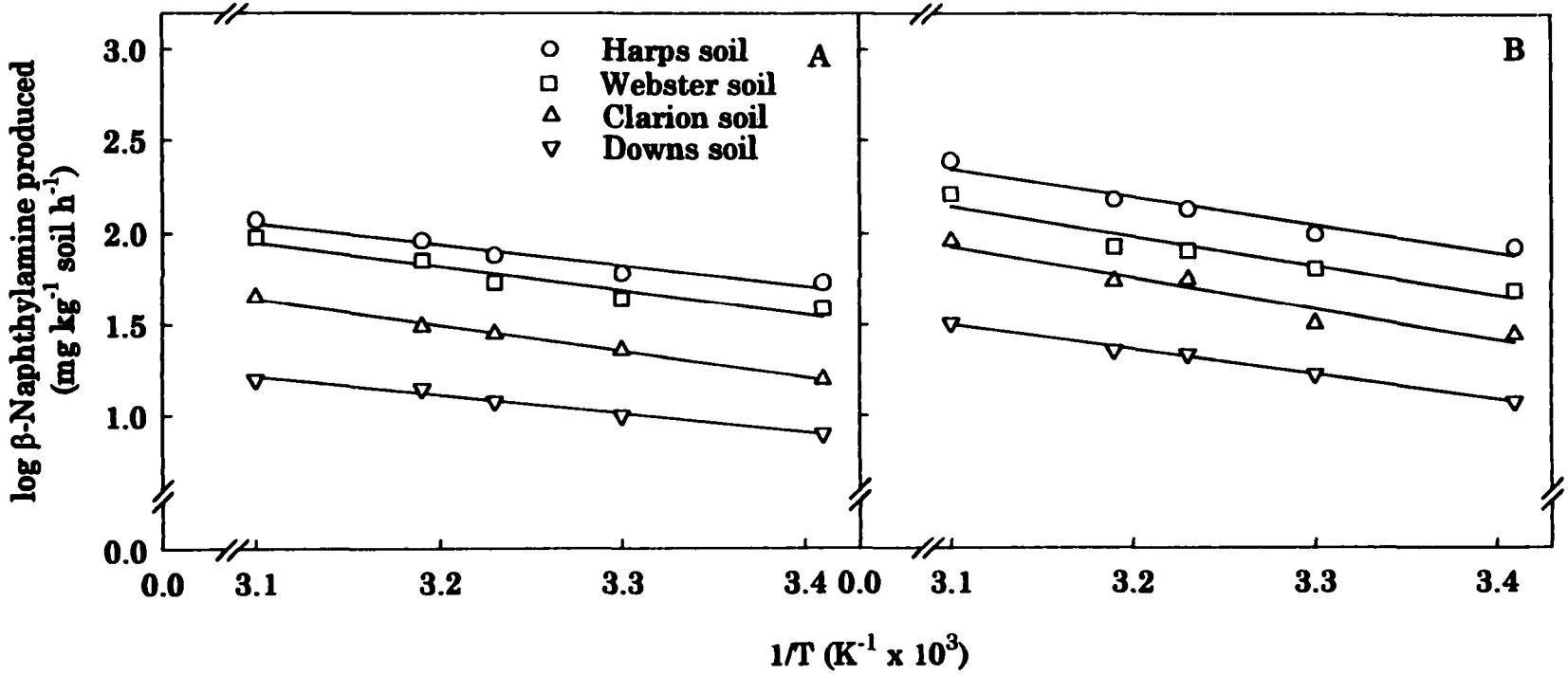


Figure 20. Arrhenius equation plot of arylamidase activity in soils. (A) Toluene; (B) no-toluene (T = absolute temperature)

without toluene ranged from 19.3 to 27.2 kJ mol^{-1} and from 26.2 to 32.4 kJ mol^{-1}, respectively (Table 11). The means of Q_{10} values for arylamidase in four soils for temperatures between 20 and 40°C ranged from 1.33 to 1.47 and 1.32 to 1.58 in presence and absence of toluene, respectively.

The enthalpy of activation values (ΔH_a) were calculated from the slope of a plot of log k/T (k = apparent values or any parameter that is proportional to the rate constant) against $1/T$ (Figure 21). The values for four air-dried soils tested ranged from 16.3 to 24.1 kJ mol^{-1} and from 23.7 to 30.1 kJ mol^{-1} for the Downs and Clarion soils in presence and absence of toluene, respectively (Table 11).

The kinetics parameters: K_m and V_{max} constants were calculated from the activity values obtained in presence and absence of toluene by using the three possible transformations of the Michaelis-Menten equation (Figures 22-24). The straight lines shown are those calculated by regression analysis and by using the following plots:

$$1/V = K_m/V_{max} \cdot 1/S + 1/V_{max} \quad \text{(Lineweaver-Burk plot)}$$

$$V = -Km \cdot V/S + V_{max} \quad \text{(Eadie-Hofstee plot)}$$

$$S/V = 1/V_{max} \cdot S + K_m/V_{max} \quad \text{(Hanes-Woolf plot)}$$

where V is the initial velocity of the reaction, K_m is the Michaelis-Menten constant, V_{max} is the maximum initial velocity and S is the substrate concentration. In the equations, both enzyme constants K_m and V_{max} may vary independently of each other under different conditions. Table 12 shows that

Table 11. Activation energies (E_a), enthalpy energies (ΔH_a) and temperature coefficient values of arylamidase activity in soils

Soil [b]	No-toluene					Toluene				
			Q_{10} of temperature (°C) indicated [a]					Q_{10} of temperature (°C) indicated [a]		
	E_a	ΔH_a	30	40	Mean	E_a	ΔH_a	30	40	Mean
	kJ mol^{-1}	kJ mol^{-1}				kJ mol^{-1}	kJ mol^{-1}			
Downs	26.2	23.7	1.80	1.35	1.58	19.3	16.3	1.25	1.40	1.33
Clarion	32.4	30.1	1.14	1.72	1.43	27.2	24.1	1.44	1.35	1.40
Webster	30.3	27.8	1.33	1.31	1.32	24.9	22.6	1.33	1.61	1.47
Harps	28.3	25.9	1.22	1.55	1.39	21.8	18.8	1.22	1.52	1.37

[a] $Q_{10} = \frac{\text{Arylamidase activity at given temperature}}{\text{Arylamidase activity at given temperature - 10°C}}$.

[b] All soils were used in air-dried conditions.

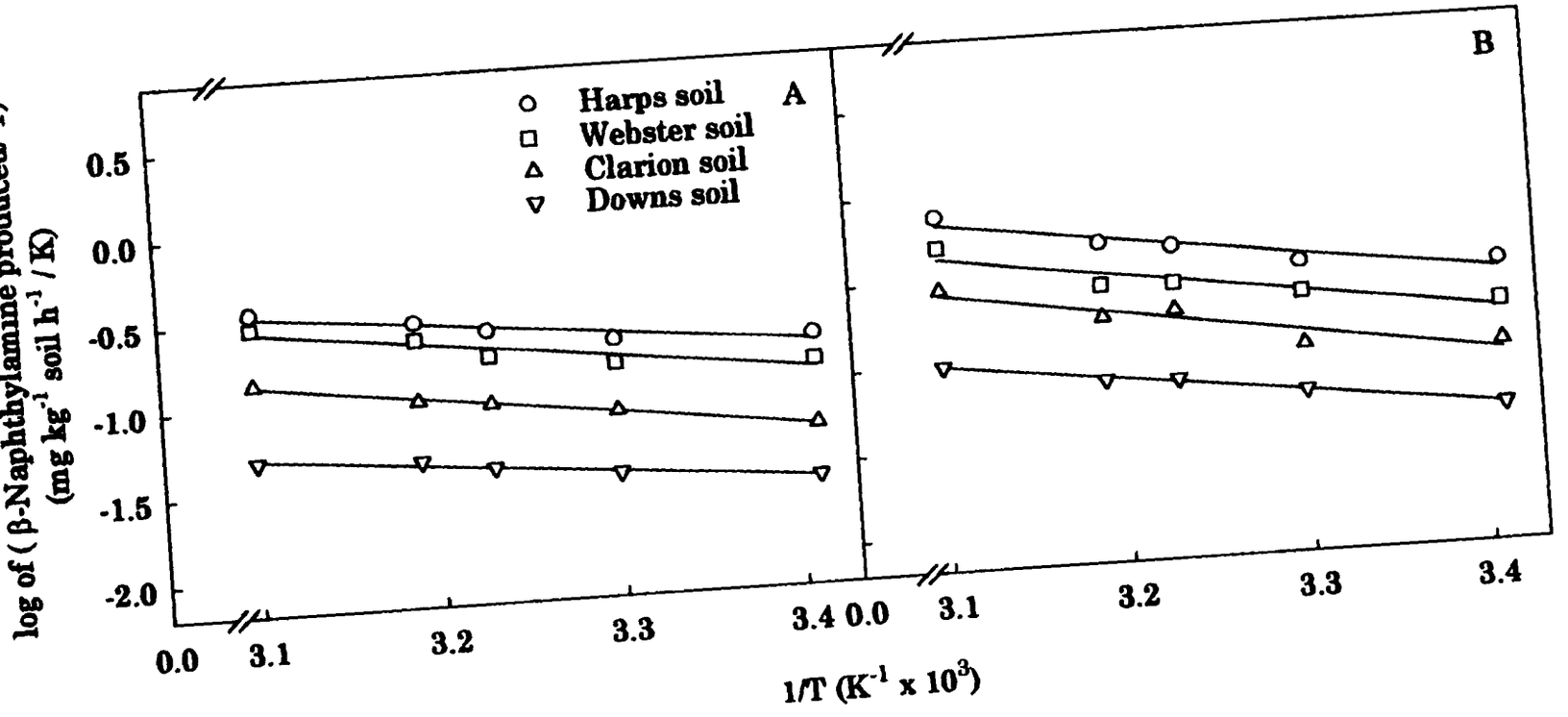


Figure 21. Equation plot of arylamidase activity in soils. (A) Toluene; (B) no-toluene (T = absolute temperature)

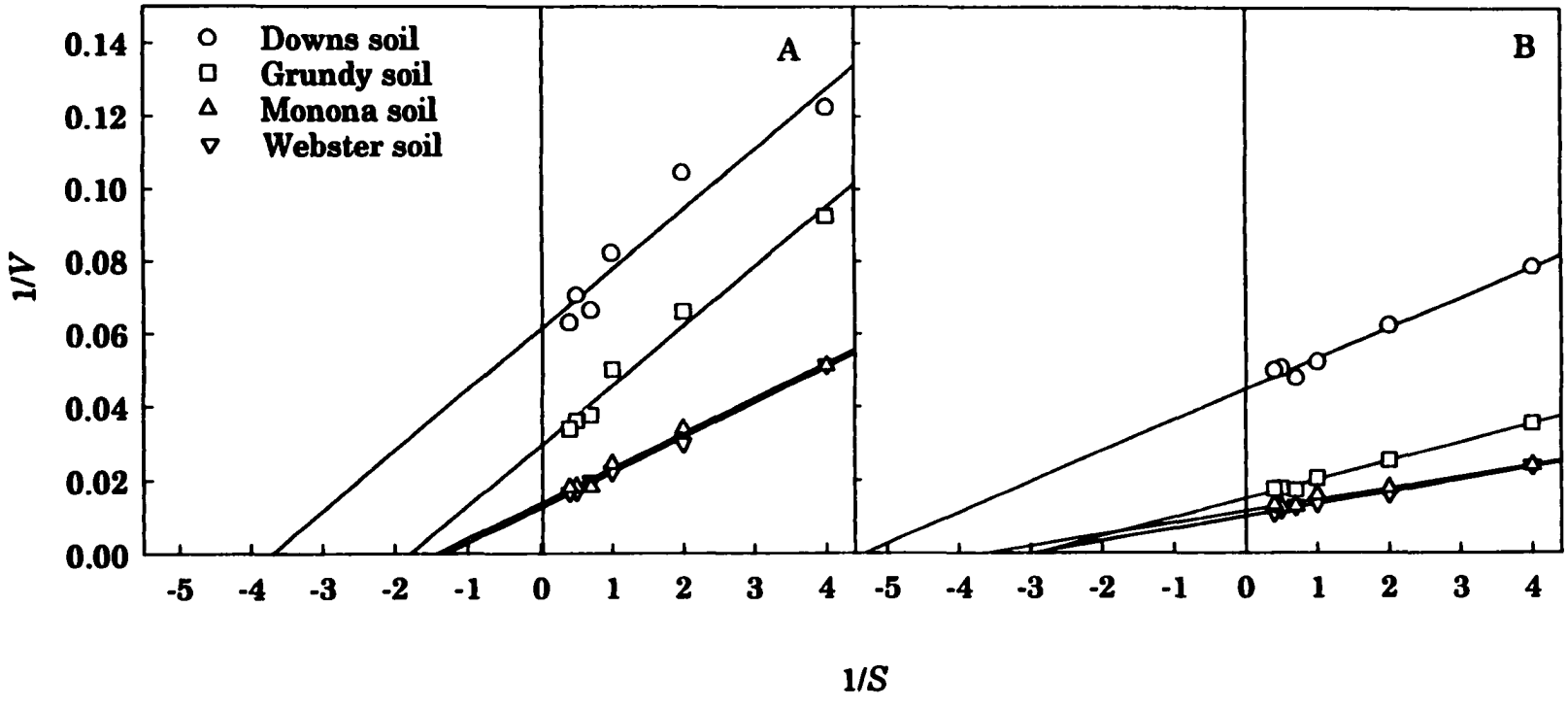


Figure 22. Lineweaver-Bulk plot of the Michaelis-Menten equation for arylamidase activity in soils (V = reaction velocity; S =substrate concentration). (A) Toluene; (B) No-toluene

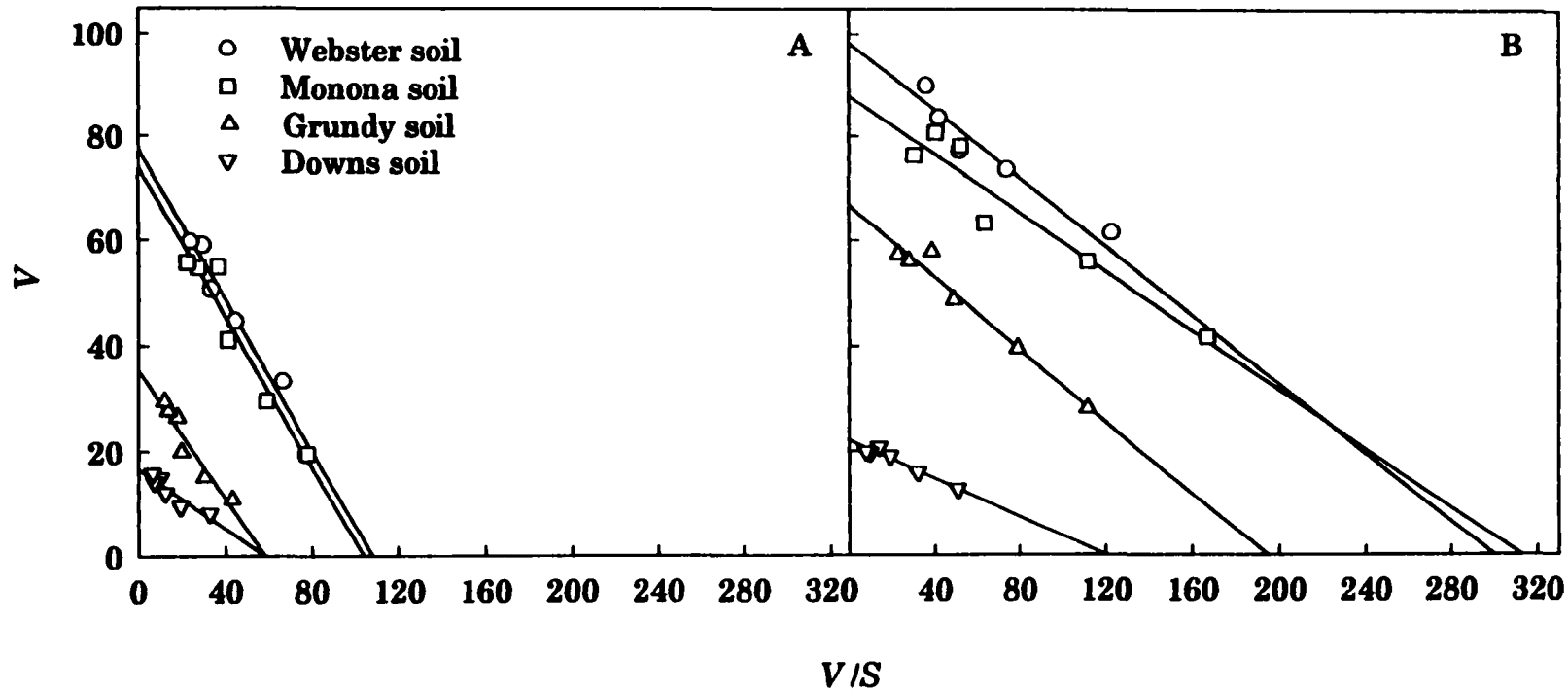


Figure 23. Eadie Hofstee plot of the Michaelis-Menten equation for arylamidase activity in soils (*V/S* = reaction velocity/substrate concentration; *V* = reaction velocity) (A) Toluene; (B) No-toluene

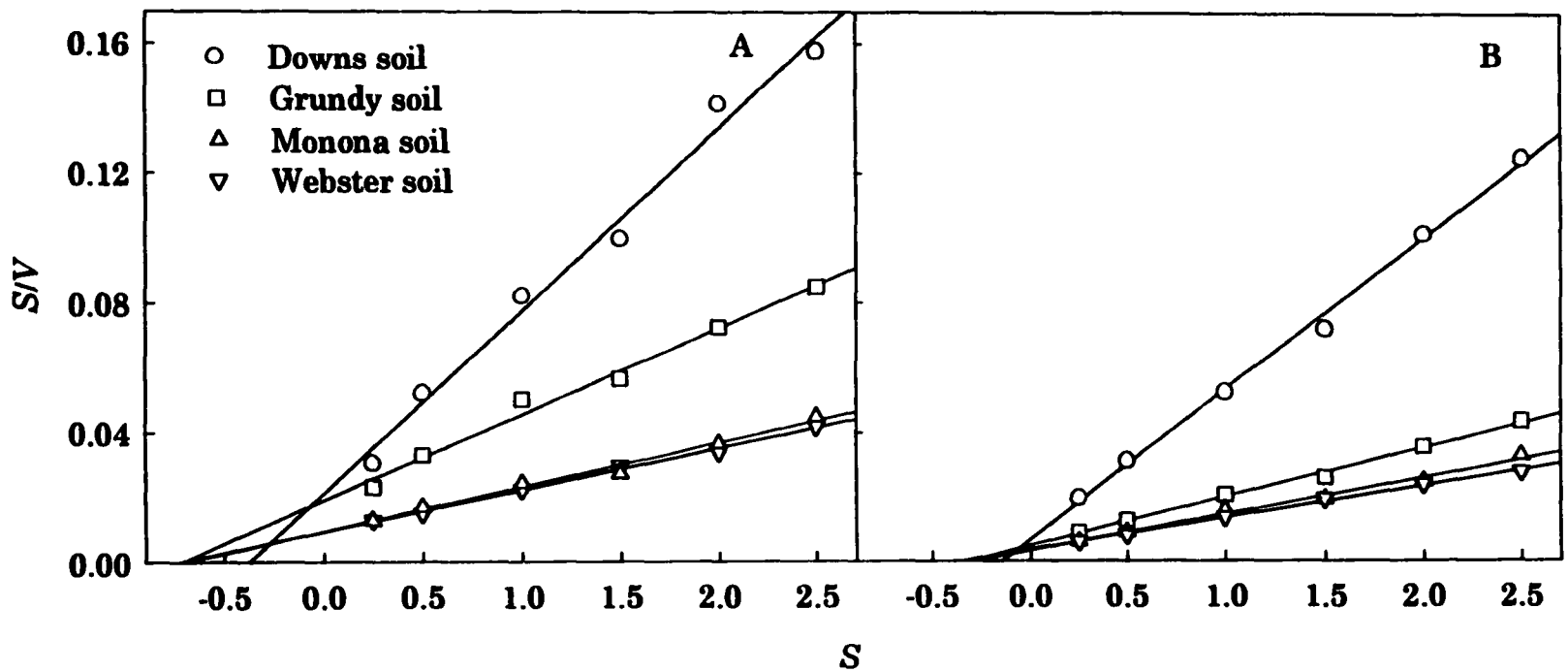


Figure 24. Hanes-Woolf plot of the Michaelis-Menten equation for arylamidase activity in soils (*S*= substrate concentration; *S*/*V*=substrate concentration/reaction velocity) (A) Toluene; (B) No-toluene

Table 12. K_m and V_{max} values of arylamidase in soils calculated from three linear transformations of the Michaelis-Menten equation in presence and absence of toluene

Michaelis-Menten transformation	Soil	Toluene [a]		No-toluene	
		K_m [b]	V_{max} [b]	K_m	V_{max}
Lineweaver-Burk plot	Downs	0.28	16	0.19	22
(1/V vs 1/S)	Grundy	0.55	35	0.35	67
	Monona	0.68	71	0.29	91
	Webster	0.77	81	0.34	100
Eadie-Hofstee plot *(V vs. V/S)*	Downs	0.29	17	0.18	22
	Grundy	0.61	35	0.34	67
	Monona	0.71	73	0.28	88
	Webster	0.72	77	0.33	98
Hanes -Woolf plot *(S/V vs. S)*	Downs	0.38	18	0.14	21
	Grundy	0.72	38	0.31	67
	Monona	0.69	73	0.29	91
	Webster	0.72	77	0.36	100

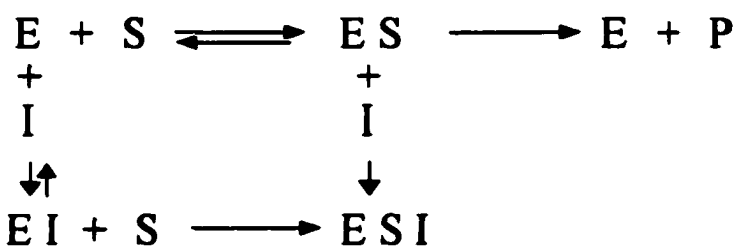
[a] Samples were always incubated with 1 mL of 8.0 *mM* L-leucine β-naphthylamine, 0.1 *M* THAM buffer (pH 8.0) and when applies 0.1 mL of toluene.

[b] K_m is expressed in m*M* and V_{max} is expressed in mg β-naphthylamine produced kg^{-1} soil h^{-1}.

addition of toluene to the soil decreased V_{max} values and increased the K_m values (decreased the affinity constant). The V_{max} values of the soils ranged from 16 to 81 and from 22 to 100 mg β-naphthylamine kg^{-1} of soil h^{-1} in presence and absence of toluene, respectively. The K_m values for arylamidase in four soils using the Lineweaver-Burk plot ranged from 0.28 to 0.77 and from 0.19 to 0.35 m*M* in presence and absence of toluene, respectively. These K_m values are one order of magnitude greater than those reported (5.5-8.7 x 10^{-2} mM) for arylamidase extracted from several human organs (Hiwada et al., 1977). Usually greater substrate concentration is required for an adsorbed enzyme to achieve the same reaction velocity as that of a free enzyme in solution.

The figure of the Lineweaver-Burk plot shows lower values of 1/*V*, corresponding to higher reaction velocity (*V*), in the soils incubated in absence of toluene compared to the same soils incubated in presence of toluene (Figure 22). The Eadie Hofstee plot shows more clearly the inhibition by toluene on arylamidase because the Y axis correspond to the velocity of the reaction (Figure 23). The Hanes-Woolf plot shows the inhibition of toluene on arylamidase by the higher values of the ratio of *S*/*V* in soils incubated in presence of toluene (Figure 24). In the latter plot, at the same substrate concentration, lower reaction velocities obtained in presence of toluene caused the ratios of *S*/*V* to be greater than those in absence of toluene.

The Lineweaver-Burk plot for arylamidase activity of the four soils assayed in the presence and absence of toluene shows that the inhibition exerted by toluene is a mixed type inhibition because there is a different X intercept ($-1/K_m$) and Y intercept ($1/V_{max}$) in the presence and absence of toluene (Figure 25). Because both constants (K_m and V_{max}) of arylamidase were affected by the presence of toluene, the results demonstrate that toluene causes a mixed type inhibition on arylamidase, which indicates that toluene inhibits both the enzyme and the enzyme-substrate complex as follows:



Previous work have reported the importance of using toluene in assay conditions for maximizing the activity of soil enzymes, because it disrupts the cell membrane causing the release of enzymes and/or the difussion of substrates and products across the cell. The reduction of the activity of arylamidase by addition of toluene to the soil in assay of arylamidase activity was unexpected. The inhibition of this enzyme by toluene could be due to the chemical structure similarity of toluene with the substrate of this enzyme L-leucine β-naphthylamide. Both toluene and L-leucine β-naphthylamide contain aromatic rings.

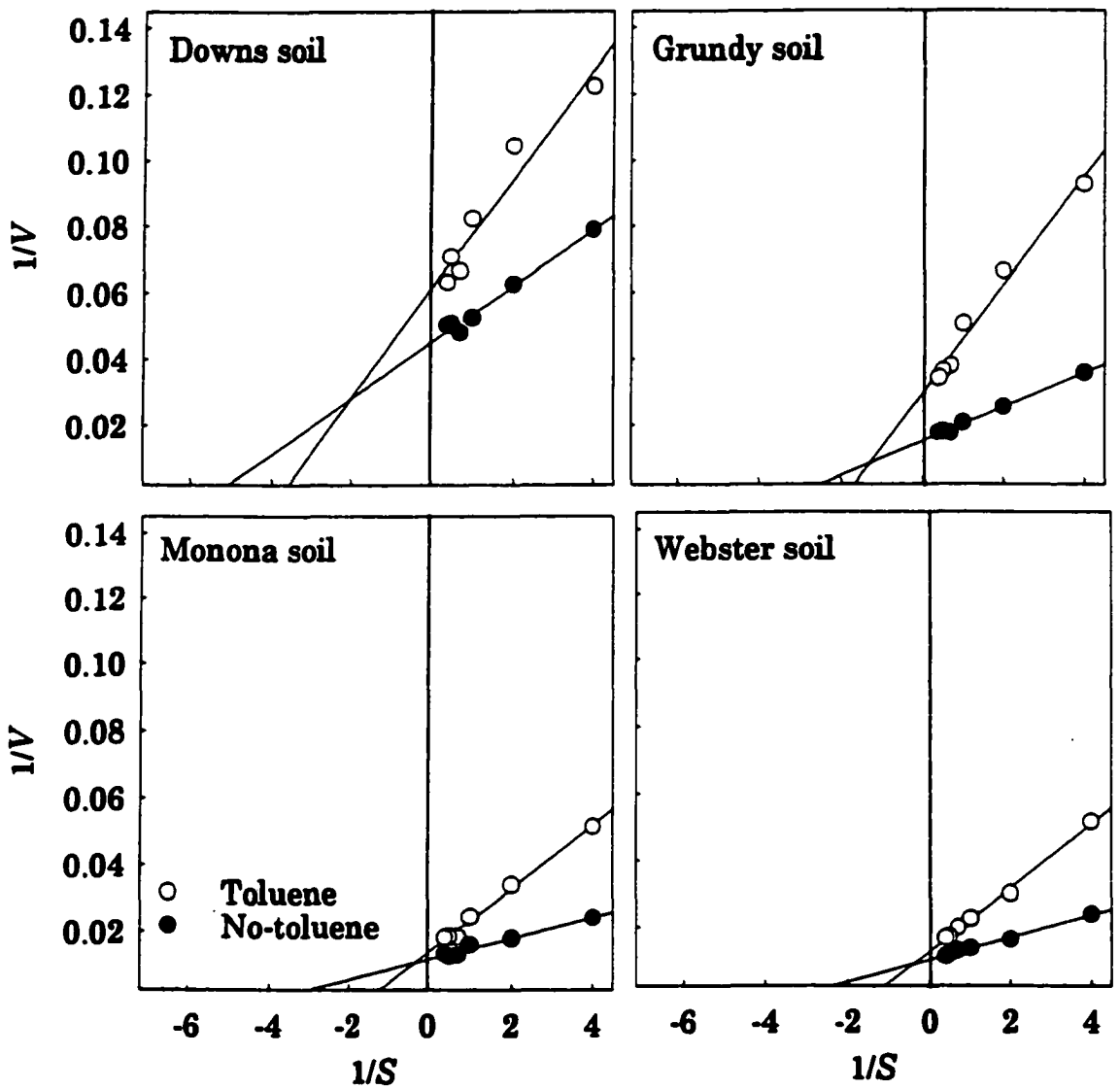


Figure 25. Lineweaver-Burk plot of the effect of toluene on arylamidase activity in soils

PART IV. SUBSTRATE SPECIFICITY OF ARYLAMIDASE IN SOILS

INTRODUCTION

The enzyme amino acid arylamidase [EC 3.4.11.2] catalyzes the hydrolysis of an N-terminal amino acid from peptides, amides or arylamides. The activity of this enzyme deserves special attention because present knowledge indicates that a variety of arylamides are present in soils (Stevenson, 1994), and that at least 14 amino acids are associated with soil organic matter (Senwo and Tabatabai, 1996). Thus, arylamidase may play an important role as an initial-limiting step in mineralization of organic N in soils. Therefore, understanding the chemical nature of the substrate controlling the activity of this enzyme in soils is important for better understanding the chemistry of N mineralization process and the ecological role of this enzyme in soils. In Part I, I showed that this enzyme is capable of hydrolyzing the neutral amino acids β-naphthylamides by using L-leucine as the amino acid moiety. The objectives of the present work were: (i) to study the specificity of this enzyme toward eight amino acid moieties, and (ii) to assess the effect of the amino acid moiety of the substrate on the kinetic and thermodynamic parameters of this enzyme in soils.

MATERIALS AND METHODS

Soils and their Properties

The soils used (Table 13) were surface samples (0-15 cm) selected to obtain a wide range of chemical and physical properties. The methods used to characterize the soils are reported in Part I.

Reagents

The substrates (Table 14) were obtained from Sigma Chemical Co, St. Louis, MO. The other reagents used are described in Part I.

Assay of Arylamidase Activity

The method of assay of arylamidase activity in soils was that described in Part I. The method involves colorimetric determination of the β-naphthylamine produced when soil is incubated with an L-amino acid β-naphthylamide in 0.1 M THAM [tris(hydroxymethyl)aminomethane] buffer (pH 8.0) at 37°C for 1 h. The β-naphthylamine produced is extracted with ethanol and converted into an azo compound by reaction with *p*-dimethylaminocinnamaldehyde, and the absorbance of the color is measured at 540 nm. In this work, the assays were performed on field-moist soils.

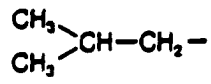
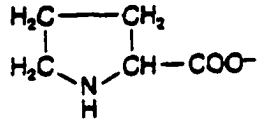
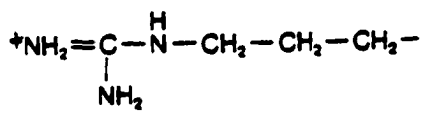
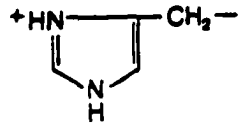
All results reported are average of duplicate assays with one control, and are reported on a moisture-free basis. Moisture was determined after drying at

Table 13. Selected properties of the soils used

Soil		pH[a]					
Series	Subgroup	H_2O	$CaCl_2$	Org. C	Total N	Clay	Sand
				g kg^{-1}			
Downs	Mollic Hapludalf	5.1	4.3	14.3	1.38	163	53
Clinton	Typic Hapludalf	6.1	5.7	16.4	1.46	318	15
Monona	Typic Hapludoll	5.7	5.1	25.4	2.54	210	58
Clarion	Typic Hapludoll	5.4	4.7	27.8	2.39	196	384
Webster	Typic Haplaquoll	6.9	6.5	32.4	3.80	280	302
Harps	Typic Calciaquoll	7.9	7.5	44.0	2.43	356	188

[a] Soil:water or soil: 0.01 *M* $CaCl_2$ ratio, 1:2.5.

Table 14. Amino acid moieties of the substrates (β-napthylamides) studied

Amino acid moiety[a]	Side chain R in $RCH(NH_3^+)CO_2^-$	pK_a's
Neutral		
L-Alanine (Ala)[b]	CH_3-	2.35, 9.87
Glycine (Gly)[b]	$H-$	2.35, 9.78
L-Leucine (Leu)[c]		2.33, 9.74
L-Proline (Pro)[b,e]		1.95, 10.64
L-Serine (Ser)[b]	$OH-CH_2-$	2.19, 9.21
Basic		
L-Arginine (Arg)[d]		1.82, 8.99, 12.48
L-Histidine (His)[d]		1.80, 6.04, 9.33
L-Lysine (Lys)[d]	$^+NH_3-CH_2-CH_2-CH_2-CH_2-$	2.16, 9.18, 10.79

[a] In parentheses are the abbreviations of the amino acids.

[b] Nonionizable polar.

[c] Nonpolar (hydrophobic).

[d] Ionizable.

[e] The structure shown is the amino acid.

105°C for 48 h. Statistical analyses were performed by using SAS version 6.11 (Barr et al., 1976). For all data points shown in the figures, the differences between duplicate values obtained in the analysis or assay were smaller than the point size. The results obtained will be discussed under subheadings according to the factors studied.

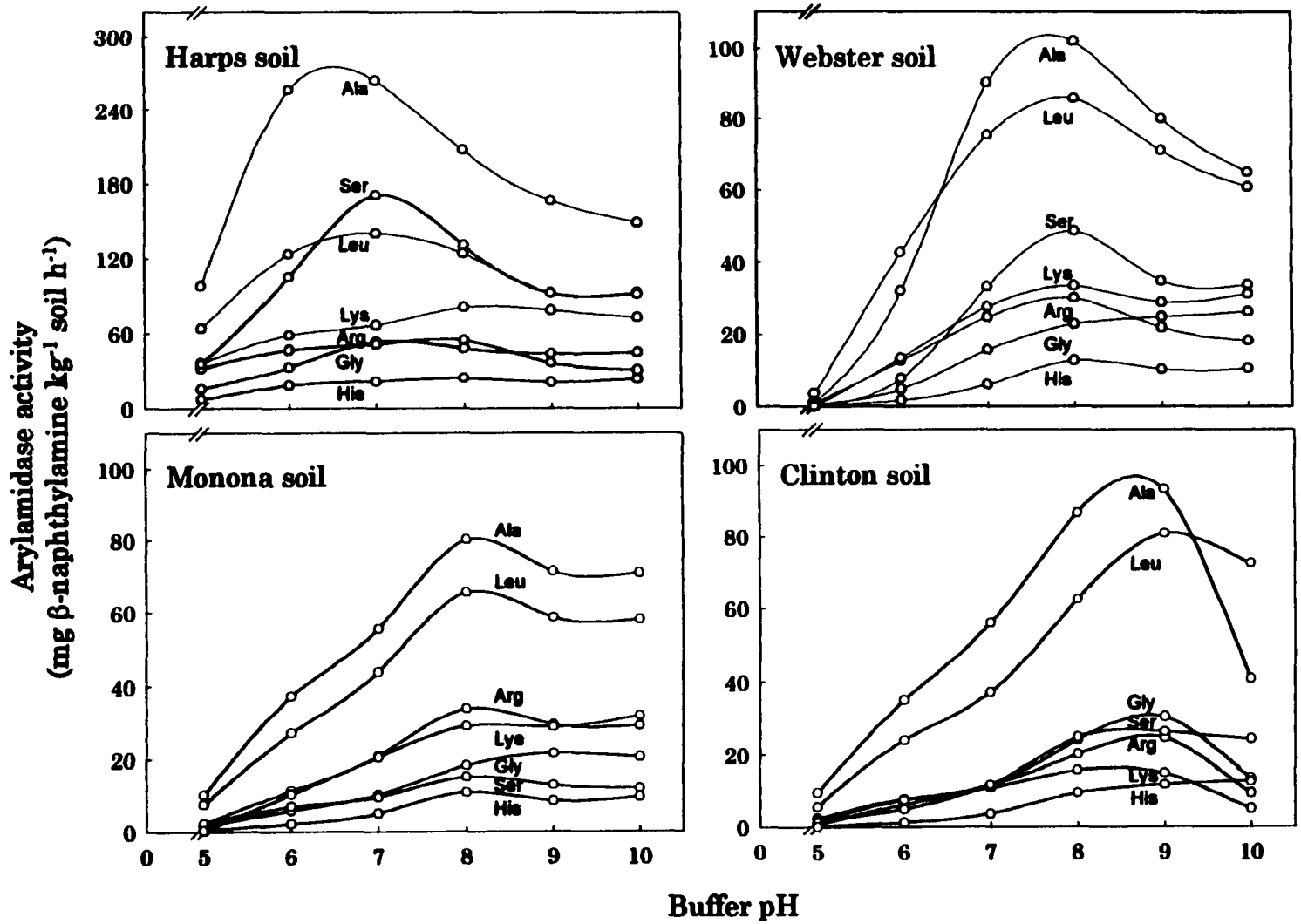
RESULTS AND DISCUSSION

Buffer pH

To ascertain the effect of the amino acid moiety of the substrate on the optimal pH of arylamidase activity, the activity was assayed at 2.0 m*M* of each of the substrates in the presence of THAM buffer at pH values ranging from 5 to 10. For preparation of the buffer solutions, see Part I.

The rates of β-naphthylamine produced from the various substrates were, in general, 8.0, but some varied from 7 to 9, depending on the soil and the amino acid moiety of the substrate studied. With the exception of the optimal pH of arylamidase activity of the Clinton soil (Figure 26), which was at pH 9.0, all the other optimal pH values were within the range (6.1-8.0). A similar pH range is reported by Appel (1974) for this enzyme purified from human and animal organs, plants, and microorganisms. The variation of the optimal pH value of this enzyme activity in soils is not surprising, because it is well known that the pH optima of enzyme in solutions are about 1.5 pH units lower than the same enzyme in soils (McLaren and Esterman, 1957). This shift in pH optimum occurs because the Bronsted acidity at the clay-organic matter surface complexes is significantly greater than in the bulk solution (Boyd and Mortland, 1990). The variation of the optimal pH with amino acid moiety of the substrate could be due to specific isozyme of arylamidase (Hiwada et al., 1980) in soils or the chemical nature (size) of the substrate molecule.

Figure 26. Effect of the amino acid moiety of the substrate on the optimal pH of arylamidase activity in four soils


Harps soil
Webster soil
Monona soil
Clinton soil
Ala
Leu
Ser
Lys
Arg
Gly
His
Arylamidase activity
(mg β-naphthylamine kg⁻¹ soil h⁻¹)
Buffer pH
0
5
6
7
8
9
10
20
40
60
80
100
120
180
240
300

Substrate Specificity

To evaluate the substrate specificity of arylamidase in soils, the activity of the enzyme was assayed in the six surface soils described in Table 13 by using 2.0 m*M* of the substrates with different amino acid moieties at the their optimal pH values (Table 15). Results showed that, in general, the rate of hydrolysis decreased with the following amino acids: Ala > Ser > Leu > Lys > Arg = Gly > His. Among the amino acid moieties used (Table 14), L- proline was not hydrolyzed in soils. Other amino acid moieties such as L-asparagine, L-tyrosine, L-glutamic acid, and L-aspartic acid were not studied because they were not soluble in water or the THAM buffer used.

Kinetic Parameters

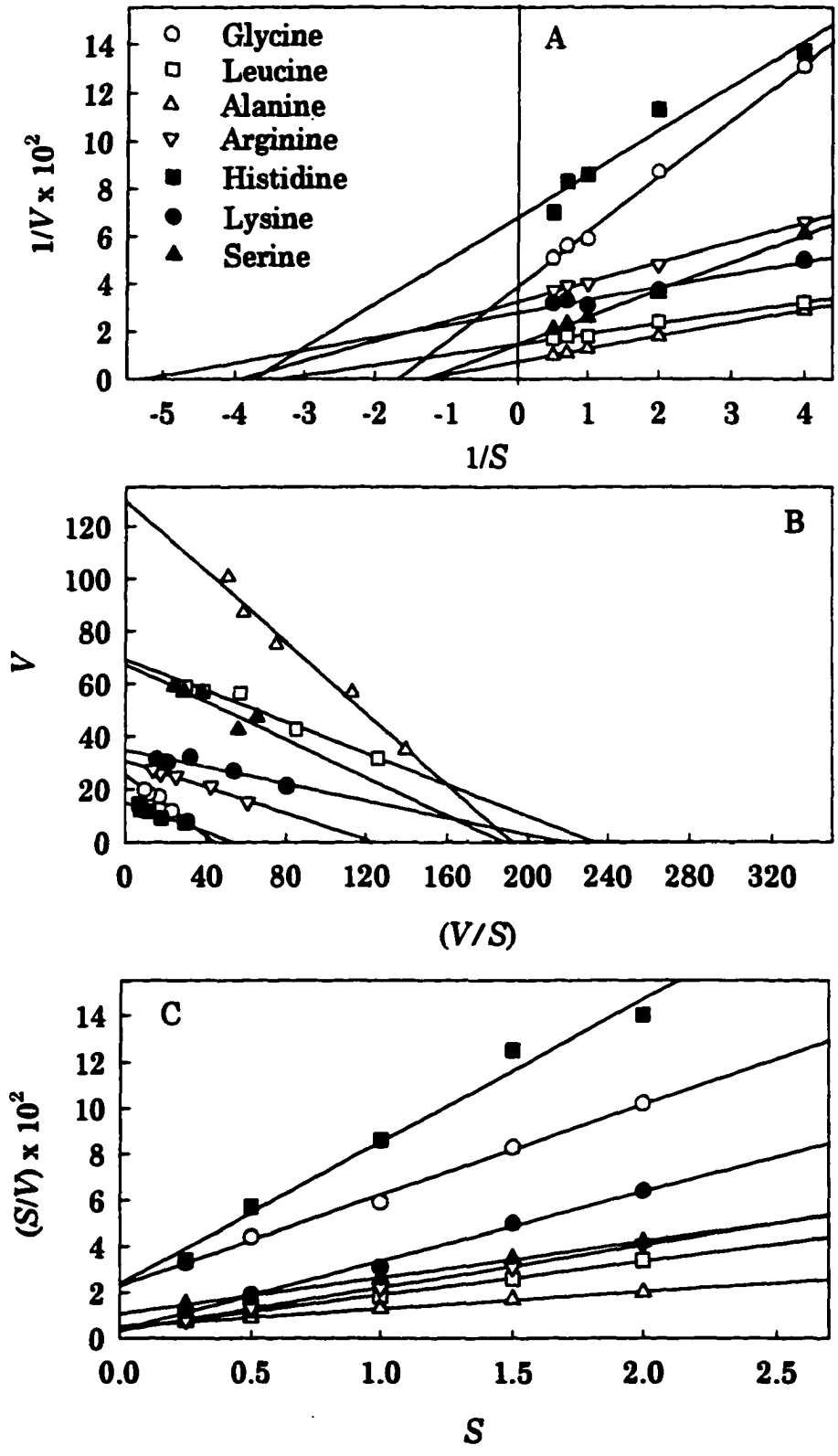
Three transformations of the Michaelis-Menten equation applied to arylamidase activity values obtained as a function of substrate concentration as affected by the amino acid moiety of the substrate for the Webster soil are shown in Figure 27. The straight lines shown are those calculated by regression analysis. Generally, the calculation of the K_m and V_{max} values by the three methods of transformation gave the same results. Each transformation gives different weight to errors in the variables (Dowd and Rigg, 1965), and this is reflected in the variation of estimated K_m and V_{max} values obtained for any soil or amino acid moiety by using the different plots. A similar observation has been reported for kinetic analysis of the reactions catalyzed by other soil

Table 15. Effect of the amino acid moiety on arylamidase activity of soils

Soil	Arylamidase activity by using the amino acid moiety specified[a]							
	Ala	Arg	Gly	His	Leu	Lys	Ser	LSD $P < 0.05$
	mg β-napthylamine kg^{-1} soil h^{-1}							
Harps	264	55	53	24	140	81	171	15
Webster	102	30	23	13	86	33	49	13
Clarion	72	10	27	7	37	13	14	4
Monona	80	34	22	11	66	29	15	7
Clinton	93	16	30	9	81	25	27	9
Downs	48	4	19	8	22	6	11	2

[a]Activity of substrate concentration = 2 m*M* at optimal pH value.

Figure 27. The three transformations of the Michaelis-Menten equation applied to arylamidase activity values as affected by the amino acid moiety of the substrate in the Webster soil. The figures represent the Lineweaver –Burk plot (A), the Eadie-Hofstee plot (B), and the Hanes-Woolf plot (C). Substrate concentration (*S*) is expressed in m*M*, and the reaction velocity (*V*) in mg β-naphthylamine released kg^{-1} soil h^{-1}


A
Glycine
Leucine
Alanine
Arginine
Histidine
Lysine
Serine
1/V x 10²
1/S
B
V
(V/S)
C
(S/V) x 10²
S

enzymes (Browman and Tabatabai, 1978; Frankenberger and Tabatabai, 1980). The variation in K_m and V_{max} values of arylamidase in soils by using the substrate linked to different amino acids (Table 16) indicates that the amino acid moiety of the substrate affected the affinity of the enzyme to its substrate.

Activation Energy, Q_{10}, and Enthalpy of Activation

Temperature dependence of enzyme-catalyzed reactions is well documented. The dependence of the rate constant on temperature (below the inactivation temperature) of an enzyme-catalyzed reaction can be expressed by the Arrhenius equation:

$$k = A \exp(-E_a/RT),$$

where k is the rate constant, A is the pre-exponential factor, E_a is the activation energy, R is the gas constant, and T is he absolute temperature in °K. The logarithmic transformation of the Arrhenius equation is expressed as follows:

$$\log k = (-E_a/2.303RT) + \log A.$$

The activation energy can be calculated from a plot of log k (or apparent values or any parameter that is proportional to the rate constant) against $1/T$ (Segel, 1975, p. 932). A representative Arrhenius equation plot for arylamidase in soils (for the Webster soil) as affected by the amino acid moiety of the substrate is shown in Figure 28. The plots of the other soils were similar. The

Table 16. Effect of the amino acid moiety of the substrate on the K_m and V_{max} values of arylamidase activity in soils calculated by using the Lineweaver-Burk plot

Amino acid moiety[b]	K_m of soil specified[a]				V_{max} of soil specified[a]			
	Clinton	Monona	Webster	Harps	Clinton	Monona	Webster	Harps
Leucine	0.23	0.30	0.30	0.57	55.3	74.6	69.7	156
Alanine	0.65	1.03	0.69	1.39	76.3	118	131	412
Glycine	0.10	0.44	0.60	0.72	23.3	26.3	25.8	55.3
Arginine	0.67	0.18	0.26	0.17	18.3	26.3	31.3	53.2
Lysine	0.27	0.32	0.19	0.12	22.0	26.3	35.5	59.2
Histidine	0.24	0.51	0.27	0.12	11.6	16.1	14.7	20.0
Serine	0.27	0.38	0.74	2.29	27.6	15.4	67.1	370

[a] K_m is expressed in mM and V_{max} in mg of β-naphthylamine released kg^{-1} soil.

[b] Amino acid attached to naphthylamine at the β-position.

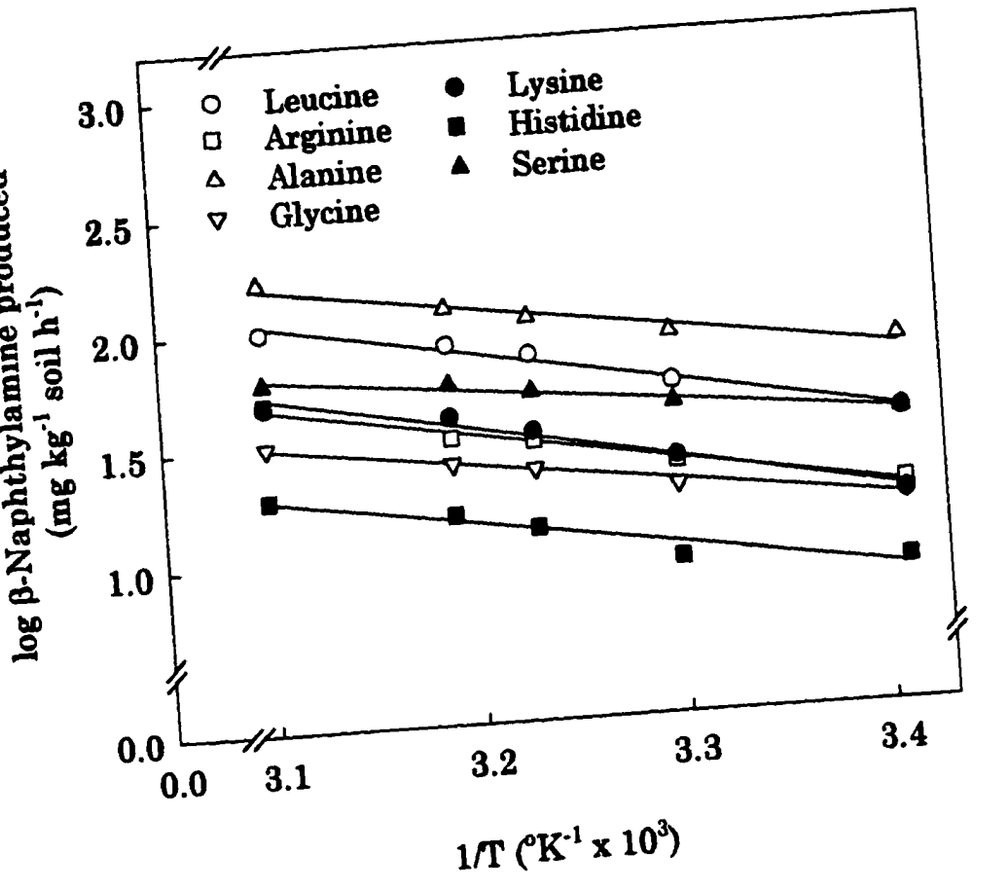


Figure 28. Arrhenius equation plot of arylamidase in the Webster soil as affected by the amino acid moiety of the substrate

activation energy values of the enzyme reaction in the four soils as affected by the amino acid moiety of the substrate are shown in Table 17. The values varied among the soils and were affected by amino acid linked to the substrate. But in general, the values are within the ranges reported for other soil enzymes (Tabatabai, 1994).

The means of Q_{10} values for arylamidase activity in four soils for temperatures between 20 and 40°C ranged from 1.04 to 1.66 for all the amino acid moieties studied (Table 18). Although the amino acid moieties significantly affected the reaction rate (Tables 15 and 16), they did not significantly affect the Q_{10} values.

The plots of calculating the ΔH_a values were similar. A representative plot is shown in Figure 29 for the effect of the amino acid moiety of the substrate on the ΔH_a values for Webster soil. Again the values varied markedly among the four soils and were affected by the amino acid linked to the β-naphthylamide (Table 17). The values of ΔH_a were lower than the values of E_a by 2.5 kJ mol^{-1} ($E_a = \Delta H_a - RT$).

Table 17. Effect of the amino acid moiety of the substrate on the activation energies (E_a) and enthalpy of activation (ΔH_a) of arylamidase activity in soils

Amino acid moiety[a]	E_a				ΔH_a			
	Clinton	Monona	Webster	Harps	Clinton	Monona	Webster	Harps
	kJ mol^{-1}				kJ mol^{-1}			
Leucine	28.5	30.4	31.6	13.0	32.4	28.0	29.3	24.7
Alanine	32.4	32.4	23.2	13.6	31.0	29.3	20.5	14.2
Glycine	30.1	30.1	20.7	21.4	27.6	27.2	18.4	18.4
Arginine	22.8	25.3	28.0	24.3	20.3	23.0	25.5	22.0
Lysine	36.2	29.1	29.1	30.8	33.1	26.6	27.8	95.5
Histidine	25.9	22.2	25.3	14.4	23.4	19.2	22.0	24.7
Serine	30.1	36.2	16.3	6.1	27.2	33.5	13.8	3.5

[a]Amino acid attached to naphthylamine at the β-position.

Table 18. Effect of the amino acid moiety of the substrate on the Q_{10} values of arylamidase activity in soils

Amino acid moiety[b]	Q_{10} at 30°C[a]				Q_{10} at 40°C[a]				Mean of Q_{10}			
	Clinto	Monona	Webster	Harps	Clinton	Monona	Webster	Harps	Clinto	Monona	Webster	Harps
Leucine	1.38	1.24	1.32	1.23	1.61	1.57	1.82	1.13	1.50	1.41	1.57	1.18
Alanine	1.38	1.34	1.17	1.23	1.74	1.69	1.44	1.18	1.56	1.52	1.31	1.20
Glycine	1.28	1.15	1.20	1.35	1.61	1.73	1.39	1.03	1.44	1.44	1.29	1.19
Arginine	1.17	1.10	1.35	1.16	1.36	1.59	1.39	1.27	1.26	1.35	1.37	1.21
Lysine	1.63	1.50	1.27	1.60	1.69	1.43	1.37	1.33	1.66	1.46	1.32	1.46
Histidine	1.33	1.25	1.13	1.44	1.63	1.40	1.67	1.23	1.48	1.33	1.40	1.33
Serine	1.15	1.29	1.21	1.10	1.73	2.00	1.34	0.99	1.44	1.64	1.27	1.04
LSD $P < 0.05$									0.28	0.46	0.32	0.25

[a] $Q_{10} = \frac{\text{Arylamidase activity at a given temperature}}{\text{Arylamidase activity at given temperature - 10°C}}$.

[b] Amino acid attached to naphthylamine at the β-position.

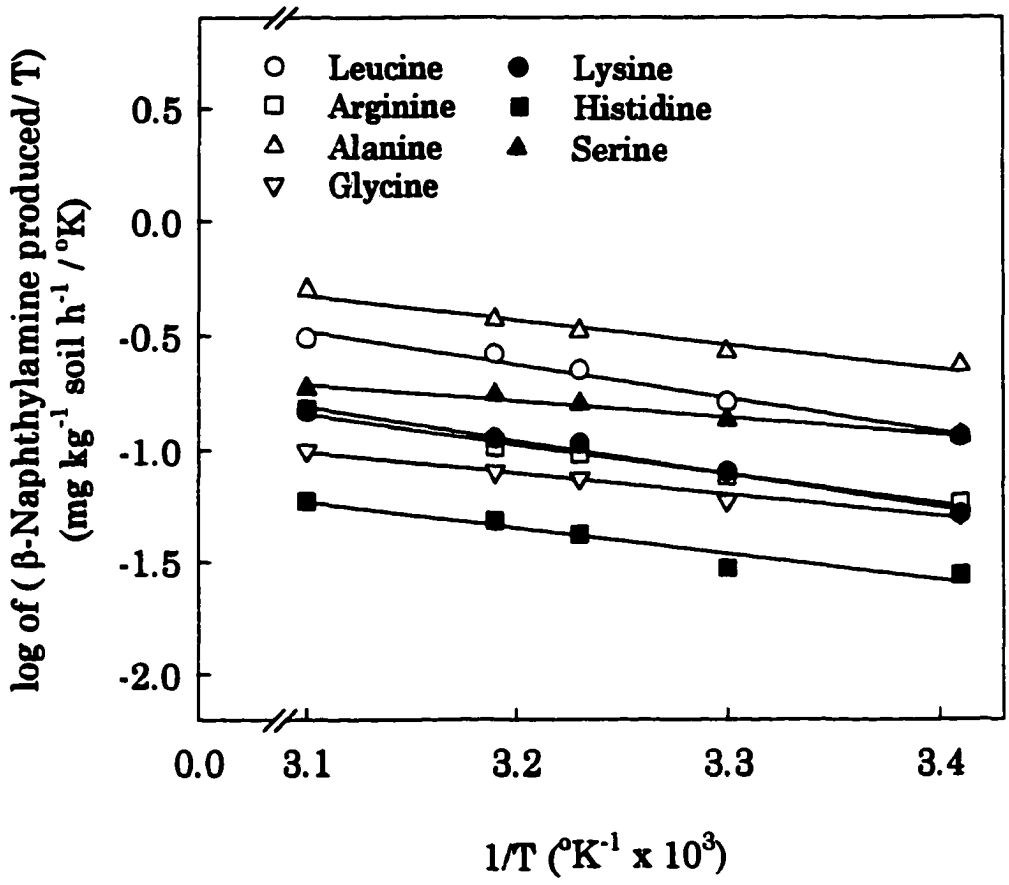


Figure 29. Equation plot of arylamidase activity in Webster soil using different amino acid moieties of the substrate

PART V. ENZYME ACTIVITIES IN A LIMED AGRICULTURAL SOIL

INTRODUCTION

Application of lime to soils normally leads to significant increases in pH and, thus, in the chemical and biochemical reactions and in microbiological processes. Such treatments result in changes in the solubility of many chemical compounds and improvement in the environment of plant roots and development (Naftel, 1965), increasing soil microbial biomass (Edmeades et al.,1981), including microbial dynamic and diversity, and, therefore, significant changes in enzyme activities (Bardgett and Leemans, 1995; Zelles et al., 1987a,b; 1990).

Recent interest in defining soil quality has focused on identifying soil properties that affect soil health and quality (Doran et al., 1994). It has been proposed that measurement of changes in soil enzyme activities may provide a useful index of changes in soil quality (Dick, 1992; Visser and Parkinson, 1992). Previous studies with limed soils have focused mostly on the changes of the activity of acid phosphatases in forest soils, because of the positive correlation between phosphate availability and soil pH (Haynes and Swift, 1988; Cepeda et al., 1991; Illmer and Schinner, 1991). Even though pH is considered one of those important properties affecting soil health and quality, its role in modifying enzymatic reactions in soil has not been demonstrated clearly, i.e., with many enzymes involving a range of soil pH. It is important to obtain a complete assessment of soil enzyme activities that reflects the changes in soil metabolic processes by using different biochemical reactions involved in nutrient cycling in

soils. Therefore, the objective of this study was to assess the effect of liming of an agricultural soil on the activities of 14 enzymes, assayed at their optimal pH values, involved in C, N, S, and P cycling in soils. The activities of the following enzymes were studied: α and β-glucosidases, α and β-galactosidases, amidase, arylamidase, urease, L-glutaminase, L-asparaginase, L-aspartase, acid and alkaline phosphatases, phosphodiesterase and arylsulfatase.

MATERIALS AND METHODS

Experimental Design

The soil used was a Kenyon loam (fine-loamy, mixed, mesic Typic Hapludoll). The experimental site was established in 1984 at the Northeast Research Center in Nashua, Iowa. Agricultural limestone from a local quarry was broadcast at the following rates: 0, 1120, 2240, 4480, 6720, 8960, 13440, and 17920 kg ECCE (Effective Calcium Carbonate Equivalent) ha^{-1}. Treatments were arranged in a randomized complete block design with four field replicates. The size of each field plot was 6 x 15 m. Corn (*Zea mays* L.) and soybean (*Glycine max L.*) were grown in alternate years, with periodic applications of fertilizers to maintain high nutrient levels of N, P, and K.

Sampling and Laboratory Analyses

Soil surface samples (0 to 15 cm) were taken after corn harvest from all the field replicates seven years after lime application by pooling 6-8 corings (7.6 cm dia). The samples were air-dried for 48 h at room temperature (22°C) and ground to pass a 2-mm sieve. Samples for chemical analyses such as organic C and organic N were ground to pass an 80-mesh (180 μm) sieve. Organic C was determined by the Mebius method (1960) and total N by a semimicro-Kjeldahl method (Bremner and Mulvaney, 1982). The pH values were measured on the < 2 mm soil samples by using a glass combination electrode (soil: water or 0.01 *M*

$CaCl_2$ ratio, 1:2.5). The activities of the enzymes were assayed on the < 2 mm air-dried samples at their optimal pH values in duplicates and one control, and are expressed on a moisture-free basis. Moisture was determined after drying at 105°C for 48 h. The enzymes studied with the reactions involved and the assay methods used are summarized in Tables 19 and 20, respectively. For all data points reported in the figures, the differences between the laboratory duplicate were smaller than the point size.

Table 19. The reactions involved in assay of the enzyme activities studied

Class/EC number	Recommended name	Reaction
Glycosidases		
3.2.1.20	α-Glucosidase	Glucoside-R + H_2O → Glucose + R-OH
3.2.1.21	β-Glucosidase	Glucoside-R + H_2O → Glucose + R-OH
3.2.1.22	α-Galactosidase	Galactoside-R + H_2O → Galactose + R-OH
3.2.1.23	β-Galactosidase	Galactoside-R + H_2O → Galactose + R-OH
Amidohydrolases and arylamidase		
3.4.11.2	Arylamidase	L-leucine β-naphthylamide → L-leucine + β-Naphthylamine
3.5.1.1	L-Asparaginase	L-Asparagine + H_2O → L-aspartate + NH_3
3.5.1.2	L-Glutaminase	L-Glutamine + H_2O → L-glutamate + NH_3
3.5.1.4	Amidase	$R\text{-}CONH_2$ + H_2O → NH_3 + R-COOH
3.5.1.5	Urease	Urea + H_2O → CO_2 + $2NH_3$
4.3.1.1	L-Aspartase	L-Aspartate + H_2O → L-aspartic acid + NH_3
Phosphatases		
3.1.3.1	Alkaline Phosphatase	RNa_2PO_4 + H_2O → R-OH + Na_2HPO_4
3.1.3.2	Acid Phosphatase	RNa_2PO_4 + H_2O → R-OH + Na_2HPO_4
3.1.4.1	Phosphodiesterase	R_2NaPO_4 + H_2O → R-OH + $RNaHPO_4$
Sulfatase		
3.1.6.1	Arylsulfatase	$ROSO_3^-$ + H_2O → R-OH + H^+ + SO_4^{2-}

Table 20. Conditions for the assay methods of the enzyme activities in soils

Class/EC number	Recommended name [a]	Assay conditions	
		Substrate [b]	Optimum pH
Glycosidases			
3.2.1.20	α-Glucosidase	*p*-Nitrophenyl-α-D glucopyranoside (10 m*M*)	6.0
3.2.1.21	β-Glucosidase	*p*-Nitrophenyl-β-D glucopyranoside (10 m*M*)	6.0
3.2.1.22	α-Galactosidase	*p*-Nitrophenyl-α-D galactopyranoside (10 m*M*)	6.0
3.2.1.23	β-Galactosidase	*p*-Nitrophenyl-β-D galactopyranoside (10 m*M*)	6.0
Amidohydrolases and arylamidase			
3.4.11.2	Arylamidase [c]	L-leucine β-naphthylamine (2.0 m*M*)	8.0
3.5.1.1	L-Asparaginase	L-Asparagine (50 m*M*)	10.0
3.5.1.2	L-Glutaminase	L-Glutamine (50 m*M*)	10.0
3.5.1.4	Amidase	Formamide (50 m*M*)	8.5
3.5.1.5	Urease	Urea (20 m*M*)	9.0
4.3.1.1	L-Aspartase [d]	L-Aspartate (200 m*M*)	8.5
Phosphatases			
3.1.3.1	Alkaline Phosphatase	*p*-Nitrophenyl phosphate (10 m*M*)	11.0
3.1.3.2	Acid Phosphatase	*p*-Nitrophenyl phosphate (10 m*M*)	6.5
3.1.4.1	Phosphodiesterase	*Bis*-*p*-Nitrophenyl phosphate (10 m*M*)	8.0
Sulfatase			
3.1.6.1	Arylsulfatase	*p*-Nitrophenyl sulfate (10 m*M*)	5.8

[a] For the methods used, see Tabatabai (1994). [b] Parentheses are substrate concentrations under assay conditions.
[c] Acosta-Martínez and Tabatabai (1999). [d] Senwo and Tabatabai (1996).

RESULTS AND DISCUSSION

To demonstrate the effect of soil pH on enzyme activities, it is essential that the percentages of organic C and N remain constant. Results showed that this condition was satisfied, and soil pH was significantly increased after seven years of the lime application, from 4.9 in the control to 6.9 in the plots treated with the highest lime application (Table 21).

Activities of Glycosidases

Glycosidases have been named according to the types of bond that they hydrolyze (Table 19). It is important to measure the activities of these enzymes in limed agricultural soils because this group of enzymes plays an important role in the degradation of organic C compounds (e.g., crop residues, biotechnology by-products, animal manures, sewage sludges) in soils (Ajwa and Tabatabai, 1994; Martínez and Tabatabai, 1997), and their hydrolysis products (sugars) are important energy sources for microorganisms in soils.

β-Glucosidase was the most predominant of the soil glycosidases followed by β-galactosidase, α -galactosidase and α-glucosidase. β- Glucosidase activity values ranged from 87 mg *p*-nitrophenol kg^{-1} soil h^{-1} in the control plots to 200 mg *p*-nitrophenol kg^{-1} soil h^{-1} in the treatments under the two highest lime application rates (13440 and 17920 kg ECCE ha^{-1}). The values of β- glucosidase activity were seven times greater than the values of the least predominant

Table 21. Effect of lime application rates on selected soil chemical properties

Lime treatment	Chemical property		
	pH[a]	Organic C	Organic N
		g kg^{-1} soil	
kg ECCE ha^{-1}			
0	4.6-5.5 (4.9)[b]	14.2-15.6 (15.0)	1.2-1.4 (1.3)
1120	4.7-5.8 (5.1)	14.7-15.3 (15.0)	1.3-1.3 (1.3)
2240	5.1-5.8 (5.1)	14.8-17.2 (15.0)	1.2-1.4 (1.3)
4480	5.3-6.2 (5.7)	15.1-15.6 (15.3)	1.3-1.3 (1.3)
6720	6.1-6.7 (6.4)	14.7-15.8 (15.2)	1.2-1.4 (1.3)
8960	6.4-6.8 (6.6)	15.3-16.5 (15.8)	1.3-1.5 (1.4)
13440	6.2-6.9 (6.6)	14.9-16.0 (15.3)	1.3-1.4 (1.3)
17920	6.7-7.0 (6.9)	14.5-16.3 (15.1)	1.2-1.4 (1.3)
LSD $P < 0.05$	0.5	NS	NS

[a] Soil: water or soil: 0.01 *M* $CaCl_2$ solution ratio, 1:2.5.

[b] Values in parentheses are averages of soil samples from four replicate field plots.

glycosidase (α-glucosidase). The predominance of β-glucosidase in soil has been well documented (Eivazi and Tabatabai, 1988; Tabatabai, 1994). The activities of the soil glycosidases were significantly correlated with soil pH (Figure 30). The correlation coefficient was the greatest for β-glucosidase ($r = 0.87^{***}$), followed by β-galactosidase ($r = 0.81^{**}$), α-glucosidase ($r = 0.56^{***}$) and α -galactosidase ($r = 0.53^{**}$). Other studies involving many soils reported no such correlation (Dick et al., 1988; Eivazi and Tabatabai, 1990). Those studies, however, compared the activities of the glycosidases in different soils where the interaction of other intrinsic soil properties may have been involved.

The results of the current study demonstrate that the significant increases in the soil pH due to lime applications led not only to an increase in the activity of β-glucosidase but also an increase in the activity of the less predominant soil glycosidases (α-glucosidase, and β and α -galactosidase). To demonstrate the sensitivity of the activities of the glycosidases to changes to soil pH, we calculated the Δ activity / Δ pH ratios for the individual glycosidase activities (Table 22). The values were 38.5 for β-glucosidase, 8.8 for β-galactosidase, 4.5 for α-galactosidase, and 4.4 for α-glucosidase. Thus, β-glucosidase is the most sensitive to soil pH changes and should be a good biochemical indicator for measuring ecological changes resulting from soil acidification.

Figure 30. Effect of soil pH on the activities of the glycosidases studied. Open symbols are results obtained for individual soil samples and solid symbols are averages obtained for the four field replicates. In calculating the regression equations, the results of the individual soil samples were used

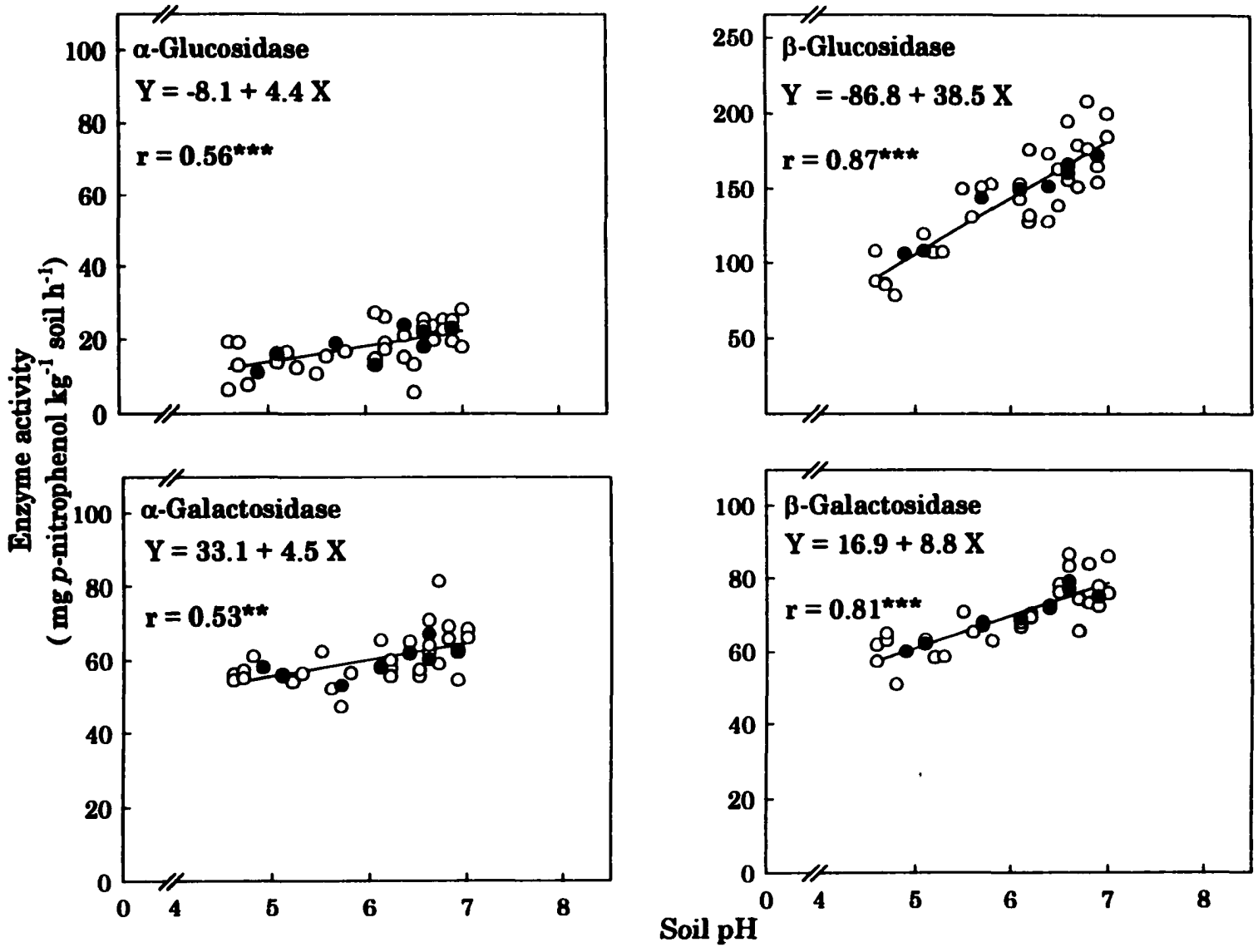

α-Glucosidase
Y = -8.1 + 4.4 X
r = 0.56***
β-Glucosidase
Y = -86.8 + 38.5 X
r = 0.87***
α-Galactosidase
Y = 33.1 + 4.5 X
r = 0.53**
β-Galactosidase
Y = 16.9 + 8.8 X
r = 0.81***
Enzyme activity
(mg p-nitrophenol kg-1 soil h-1)
Soil pH

Table 22. Correlation coefficients (r) and slopes of linear relationships of soil pH and enzyme activities

Enzyme	Enzyme activity vs soil pH [a] r	Δ Enzyme activity[b] / Δ soil pH
Glycosidases		
α-Glucosidase	0.56***	4.4
β-Glucosidase	0.87***	38.5
α-Galactosidase	0.53 **	4.5
β-Galactosidase	0.81***	8.8
Amidohydrolases and arylamidase		
Arylamidase	0.74***	9.0
L-Asparaginase	0.84***	15.8
L-Glutaminase	0.73***	107
Amidase	0.61***	14.4
Urease	0.71***	6.2
L-Aspartase	0.80***	1.0
Phosphatases		
Alkaline Phosphatase	0.89*** [c] (0.95***)[d]	97.0 [c] (25.6)[d]
Acid Phosphatase	-0.69***	-35.0
Phosphodiesterase	0.89*** [c] (0.91***)[d]	39.4 [c] (12.1)[d]
Sulfatase		
Arylsulfatase	0.66***	11.2

[a] ** $P < 0.01$, *** $P < 0.001$.

[b] All activities are expressed in mg product released kg^{-1} soil h^{-1}.

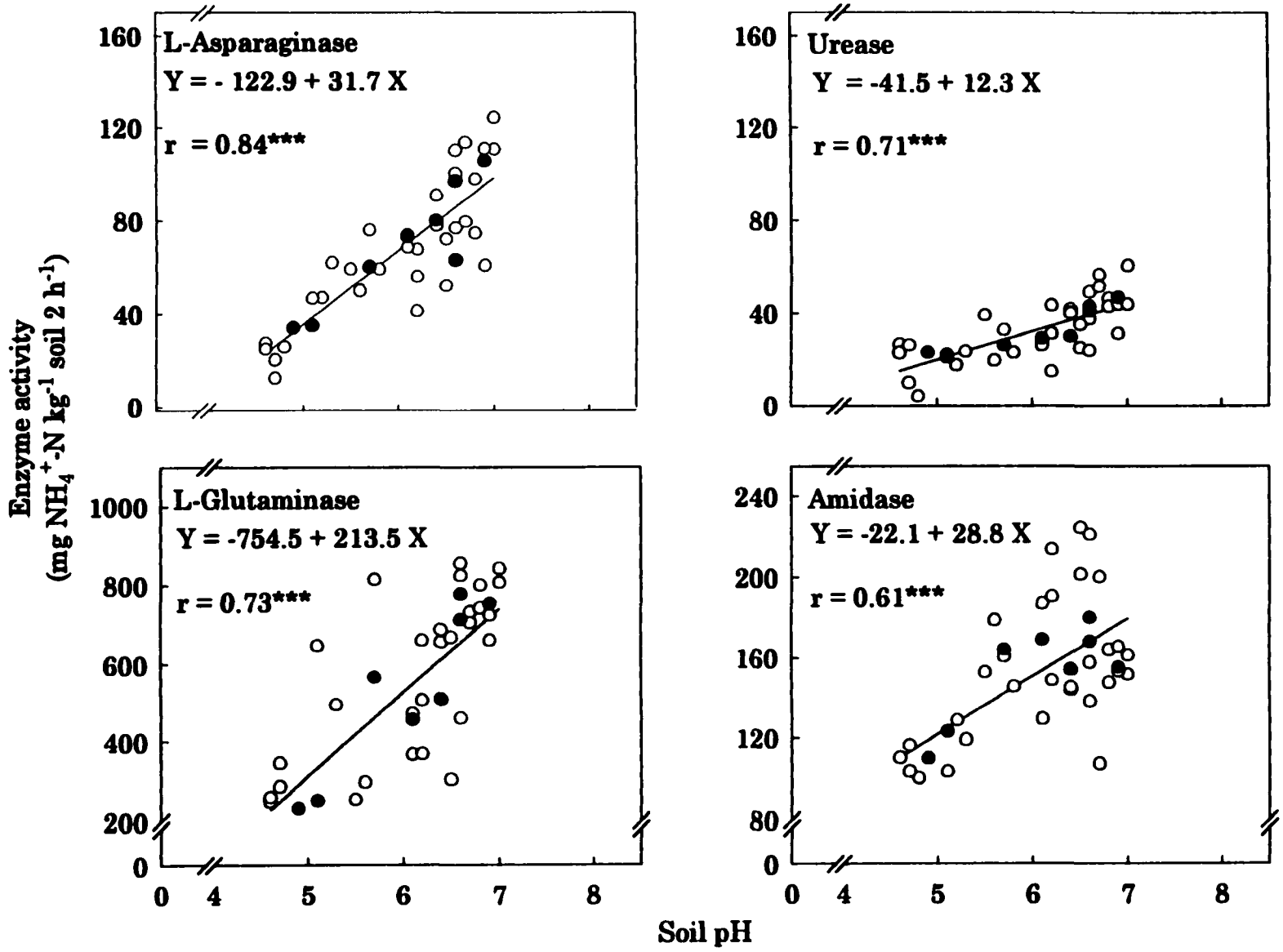
[c] Linear regression for soils with pH > 6.0.

[d] Second linear regression values.

Activities of Amidohydrolases and Arylamidase

The assessment of the activities of the enzymes involved in N cycling in soils may provide more insight into the N mineralization cycle as each enzyme catalyzes a specific reaction (Tabatabai, 1994). To my knowledge, little information is available on the effect of liming on the activities of those enzymes in soils. In addition to the well-known amidohydrolases, I studied the effect of liming on the activity of arylamidase in soils. Therefore, I studied the effect of liming on the activities of five enzymes involved in organic N hydrolysis in soils. Results showed that L-glutaminase activity was the most predominant of the N cycling enzymes, followed by the activities of amidase, L-asparaginase, arylamidase, urease and L-aspartase. The activity of L-glutaminase ranged from 160 in the control to 840 mg NH_4-N released kg^{-1} soil $2h^{-1}$ in the treatment under the highest lime application rate (17920 kg ECCE ha^{-1}). These results support the previous finding that most of the NH_4^+ released in soils is derived from the hydrolysis of amide (asparagine, aspartase and glutamine) functional groups of soil organic matter (Sowden, 1958). The activities of these soil enzymes were significantly correlated ($P < 0.001$) with soil pH (Figure 31). L-Asparaginase showed the greatest correlation coefficient ($r = 0.84^{***}$), followed by L-aspartase ($r = 0.80^{***}$), arylamidase ($r = 0.74^{***}$), L-glutaminase ($r = 0.73^{***}$), urease ($r = 0.71^{***}$), and amidase ($r = 0.61^{***}$) (Figures 31 and 32). The Δ activity / Δ soil pH ratios were 107 for L-glutaminase, 15.8 for L-asparaginase, 14.4 for amidase, 9.0 for arylamidase, 6.2 for urease and 1.0 for L-

Figure 31. Effect of soil pH on the activities of L-asparaginase, urease, L-glutaminase and amidase. For the symbols and regression equation, see the caption of Figure 30


L-Asparaginase
Y = - 122.9 + 31.7 X
r = 0.84***
Urease
Y = -41.5 + 12.3 X
r = 0.71***
L-Glutaminase
Y = -754.5 + 213.5 X
r = 0.73***
Amidase
Y = -22.1 + 28.8 X
r = 0.61***
Enzyme activity
(mg NH4+-N kg-1 soil 2 h-1)
Soil pH

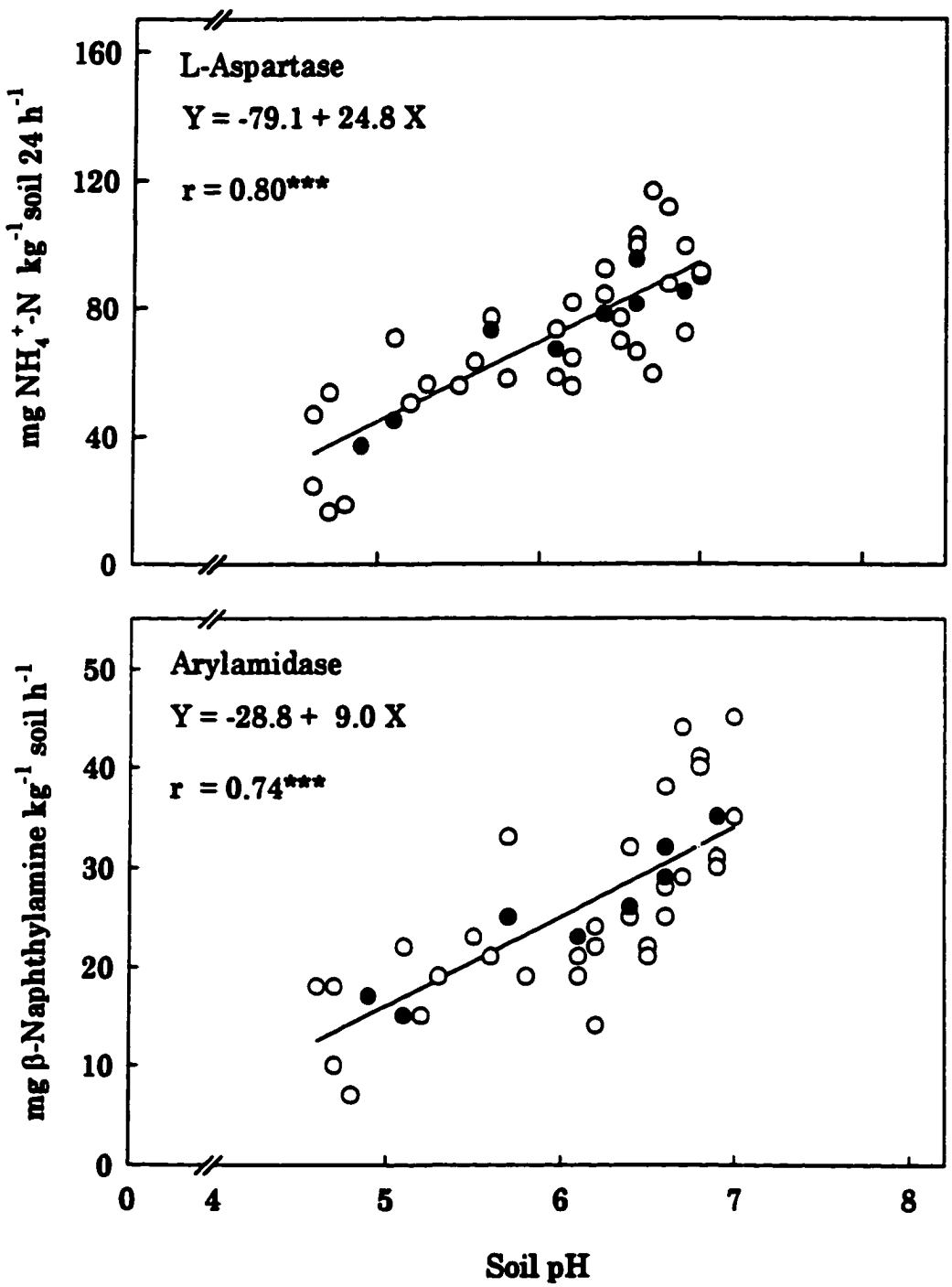


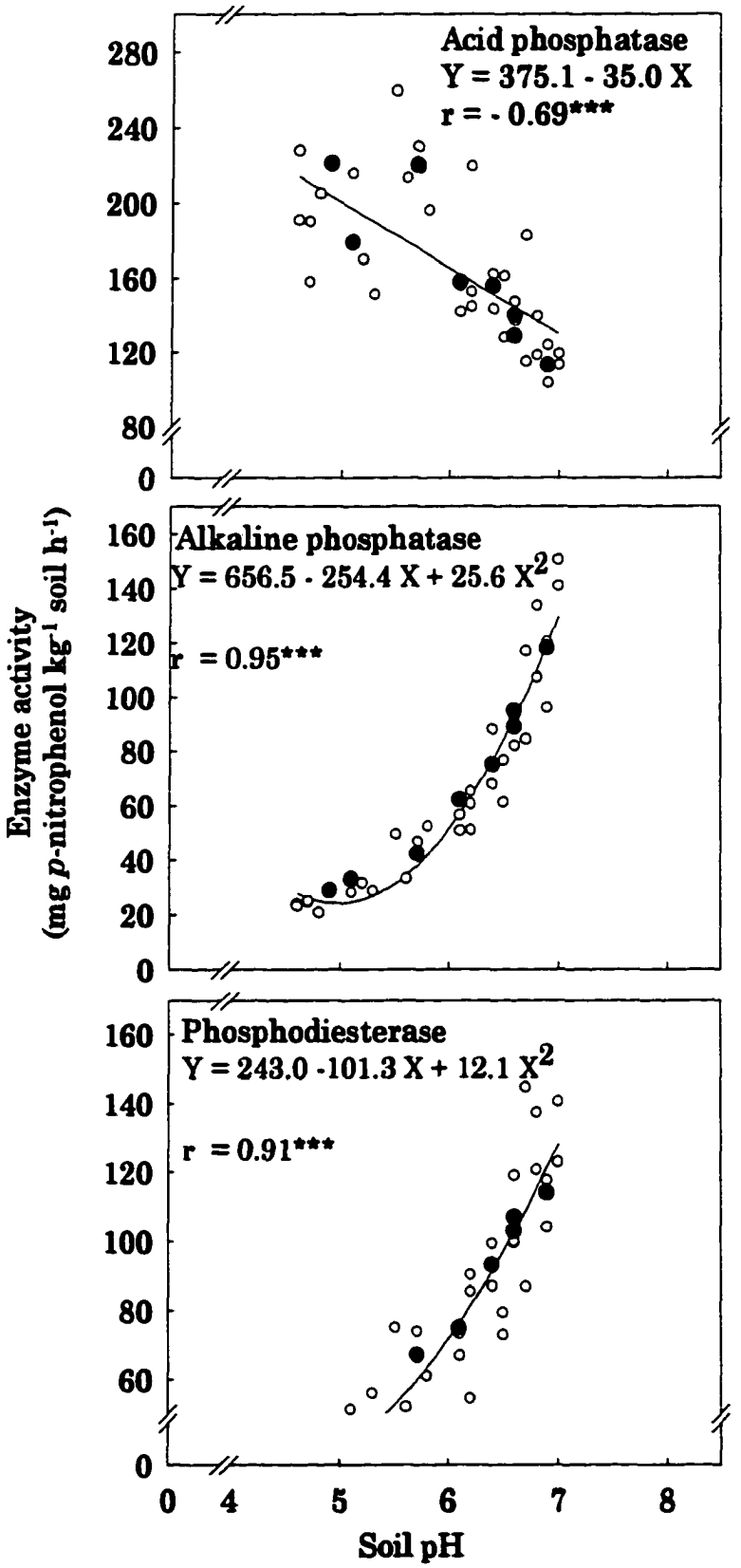
Figure 32. Effect of pH on the activities of L-aspartase and arylamidase. For the symbols and regression equation, see Figure 30

aspartase. Results showed that L-glutaminase was not only the most sensitive enzyme to soil pH changes among those involved in N cycling but also the most sensitive enzyme among the 14 enzymes included in this study (Table 22).

Activities of Phosphatases and Arylsulfatase

Phosphatases is a general name for a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowski, 1961). The study of this group of enzymes was important because the soil pH was significantly affected by lime application, and because it has been suggested that the rates of synthesis, release, and stability of acid and alkaline phosphatases by soil microorganisms are dependent on the soil pH (Eivazi and Tabatabai, 1977; Juma and Tabatabai, 1977; Tabatabai, 1994). Results showed that acid phosphatase was predominant in the control plots (pH 4.9) and that liming increased the activities of the alkaline phosphatase and phosphodiesterase (Figure 33). These results support the findings of Juma and Tabatabai (1978) that acid phosphatase is predominant in acid soils and that alkaline phosphatase is predominant in alkaline soils. The correlation coefficient between the activities of acid phosphatase or alkaline phosphatase or phosphodiesterase and soil pH were –0.69***, 0.89*** and 0.89***, respectively (Table 22). The increase in the activity of alkaline phosphatase may demonstrate the effect of lime applications on the size of the soil microbial population as this enzyme is not present in higher plants, and,

Figure 33. Effect of soil pH on the activities of phosphatases. For the symbols and the regression equation, see the caption of Figure 30


Acid phosphatase
Y = 375.1 - 35.0 X
r = - 0.69***
Alkaline phosphatase
Y = 656.5 - 254.4 X + 25.6 X2
r = 0.95***
Phosphodiesterase
Y = 243.0 -101.3 X + 12.1 X2
r = 0.91***
Enzyme activity
(mg p-nitrophenol kg-1 soil h-1)
Soil pH
0
4
5
6
7
8
0
80
120
160
200
240
280
20
40
60
100
140

thus, its activity is derived totally from microorganisms (Dick et al., 1983; Juma and Tabatabai, 1988 a,b,c).

The different response of acid and alkaline phosphatases to lime application supports the previous findings that phosphatases are inducible enzymes and the intensity of their excretion by plant roots and microorganisms is determined by their requirement for orthophosphate, which is affected by soil pH (Skujins, 1976). The Δ activity/ Δ soil pH ratios for the phosphatases were 97 for alkaline phosphatase, 39.4 for phosphodiesterase, and –35.0 for acid phosphatase (Table 22).

Arylsulfatase is the enzyme involved in the hydrolysis of arylsulfate by fission of the O-S bond (Spencer, 1958). This enzyme is believed to be involved in mineralization of ester sulfate in soils (Tabatabai, 1994). The means of activity of this enzyme in the four field replicates ranged from 92 to 114 mg of *p*-nitrophenol released kg^{-1} soil h^{-1} in the control plots and at the highest rate of lime application, respectively. The activity of this enzyme was significantly correlated with soil pH ($r = 0.66^{***}$) (Figure 34). The Δ activity/ Δ soil pH ratios for arylsulfatase was 11.2 (Table 22).

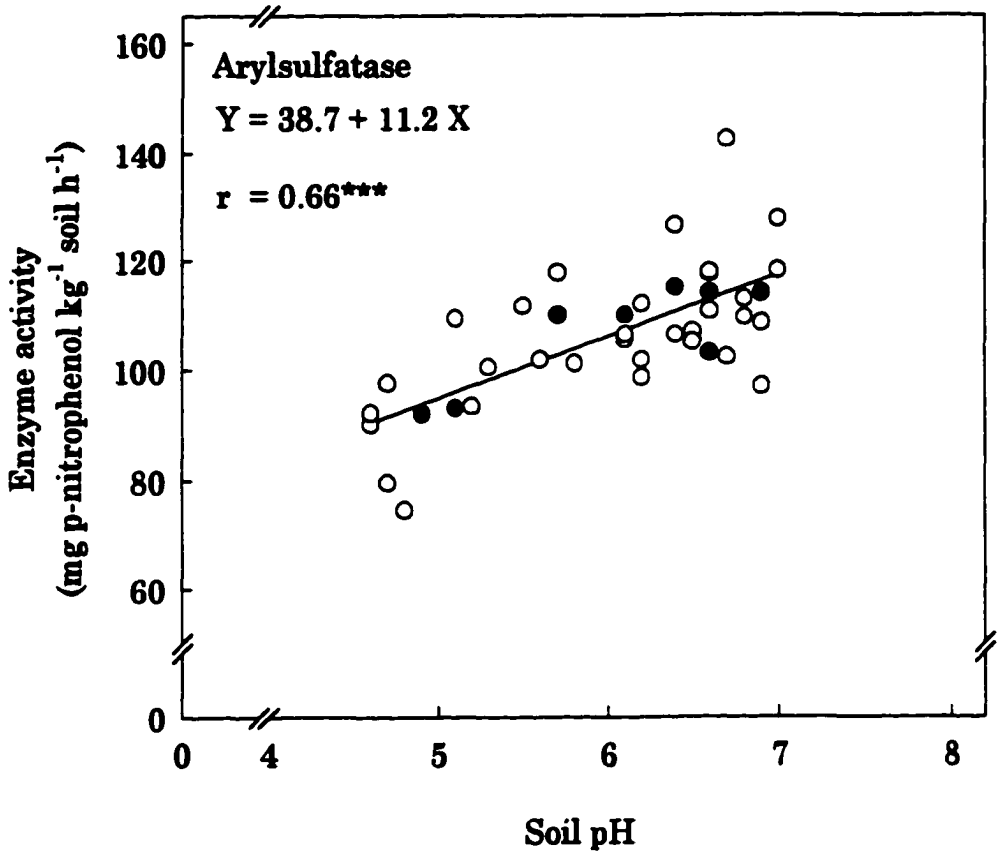


Figure 34. Effect of soil pH on the arylsulfatase activity. For the symbols and regression equation, see the caption of Figure 30

PART VI. EFFECT OF TILLAGE AND RESIDUE MANAGEMENT ON ARYLAMIDASE ACTIVITY IN SOILS

INTRODUCTION

Recently, the attention has focused on improving the common agricultural practices of the past years, including tillage and crop-residue management, to minimize the degradation of soil resources. Conventional tillage consists of moldboard plow or chisel plow to bury plant residues, and of the additional tillage operations for seedbed preparation and weed control. Plowing leads to the mixing of the soil surface to a depth that depends on the equipment used. No-till consists on limited tillage for seed placement complemented by weed control with herbicides and on the accumulation of the crop residues on the soil surface as mulch. No-till is preferred by farmers because it improves the soil structure and aggregation and, thus, reduces soil erosion and water evaporation, and increases water infiltration and the storage of soil water (Doran, 1980a,b; Tracy et al., 1990).

The soil disturbance from different tillage and residue management practices may lead to significant changes in the soil chemical, biological and biochemical properties and, thus, affects the composition, distribution and activities of the soil microbial communities and the production of enzymes (Doran, 1980a,b; Dick, 1994; Magan and Lynch, 1986). The distribution of organic matter and nutrients in a soil profile is changed when no-tillage practices are adopted. Studies have reported the accumulation of organic C, N, and inorganic nutrients such as NO_3, SO_4, and PO_4 at the soil surface under

reduced tillage and crop residue placement (Havlin et al., 1990; Tracy et al., 1990; Murata and Goh, 1997). Several researchers have reported the increases in the microbial biomass in the soil surface by reducing tillage intensity (Carter, 1986; Doran, 1987; Angers et al., 1993).

The microbial biomass has a key role in reflecting the changes in the soil quality because it is both a source of labile nutrients and an agent of the transformation and cycling of the soil organic matter by the activities of the living microbial communities and their accumulated enzymes in soils. Dick (1984) reported that the activities of acid phosphatase, alkaline phosphatase, arylsulfatase, invertase, amidase, and urease were increased in the top 7.5 cm of soil with no-tillage compared to conventional tillage. Soil acid phosphatase and dehydrogenase activities were found to be higher in the top 7.5 cm depth in no-tilled treatments compared to the conventional tillage treatments (Doran, 1980b). Angers et al. (1993) reported greater alkaline phosphatase activity under no-till, intermediate under chisel plow, and least under moldboard plow. Deng and Tabatabai (1996a,b; 1997) reported that the activities of amidohydrolases (urease, amidase, L-asparaginase, and L-glutaminase), glycosidases (α- and β- glucosidases and α- and β- galactosidases) and phosphatases (alkaline and acid phosphatase and phosphodiesterase) were generally greater under no-till and double mulch plots compared to the other combinations of tillage-crop residue treatments used. However, there is no information available about the effect of tillage and residue management on the

activity of that I recently detected in soils, namely arylamidase (see Part I).

Understanding the effects of tillage and crop residue management on the activity of arylamidase (involved in N mineralization) would lead to better strategies for the manipulation of such management practices for improving soil quality, and the fertility and productivity. Therefore, the objective of this part was to study the effect of tillage and residue management on the activity of arylamidase in soils.

MATERIAL AND METHODS

Treatments Description

The tillage and residue management study was initiated in 1981 at the Lancaster Experiment Station in Wisconsin. The soil is a Palsgrove silt loam (fine-silty, mixed, mesic Typic Hapludalfs) that contained 17% of clay and 6% of sand. The tillage systems studied were moldboard plow, chisel plow and no-till. The crop residue treatments applied were the following:

Bare = Crop residue removed before planting (no prior tillage).

Normal = No removal or addition of crop residue.

Mulch = Crop residue added after primary tillage.

Double mulch = Crop residue addition to achieve 2x normal level of crop residue.

For convenience, the following abbreviations will be used in plotting the results:

NTB = no-till / bare

NTN = no-till / normal

NT2M = no-till / double mulch

CPN = chisel plow / normal

CPM = chisel plow / mulch

MPN = moldboard plow / normal

MPM = moldboard plow / mulch

The residues in all cases were corn stalks. The area had been in continuous corn since 1971 (at least 10 years before the study) and was maintained in continuous corn during the study. The design and location of the experiment was described in detail by Swan et al. (1987a,b).

Soil Sampling and Laboratory Analyses

The soil samples used in this study were taken from four replicated plots of all the treatments in May 1991 (10 years after the crop residue treatments were initiated) at the 0 to 15 cm depth. Samples of the no-till / 2x mulch treatments were also taken at the depth intervals: 0 to 5, 5 to 10, and 10 to 15 cm. A subsample of each soil sample was air-dried and ground to pass a 2-mm sieve. The pH values of these soil samples (Table 23) were measured on the < 2 mm soil by using a glass combination electrode in both water and 0.01 *M* $CaCl_2$ solutions (soil: solution ratio, 1:2.5). The organic C of the soil samples (Table 24) was determined by the Mebius method (1960) on <80 mesh (177 μm) samples.

Assay of Arylamidase Activity

To assay the activity of arylamidase in the samples, the method described in Part I was used. The procedure for preparing the calibration curve and for preparing the soil samples is also described in Part I.

Table 23. Effect of tillage and residue management on soil pH[a]

Treatments	Rep I		Rep II		Rep III		Rep IV		Mean[b]		LSD[b] $P < 0.05$	
	water	$CaCl_2$	water	$CaCl_2$	water	$CaCl_2$	water	$CaCl_2$	water	$CaCl_2$	water	$CaCl_2$
No Till/Bare	7.2	6.3	7.1	6.4	6.5	5.8	5.1	4.3	6.9	6.2	0.03	0.05
No Till/Normal	6.9	6.2	7.2	6.5	6.6	5.8	5.0	4.2	6.9	6.2	0.07	0.02
No Till/ 2X Mulch	6.8	6.1	7.0	6.3	6.4	5.6	5.1	4.2	6.7	6.0	0.10	0.04
Chisel/Normal	7.1	6.4	7.1	6.5	7.0	6.3	4.9	4.2	7.1	6.4	0.02	0.03
Chisel/ Mulch	7.1	6.5	7.1	6.5	7.1	6.5	5.2	4.3	7.1	6.5	0.02	0.06
Moldboard Plow / Normal	6.9	6.2	6.9	6.2	7.0	6.2	4.9	4.1	6.9	6.2	0.02	0.05
Moldboard Plow / Mulch	6.7	6.1	7.0	6.4	7.0	6.5	6.0	5.0	6.9	6.3	0.06	0.11
LSD $P < 0.05$	0.04	0.03	0.06	0.03	0.03	0.03	0.03	0.02	0.4	0.4		
Depth of No Till / 2x Mulch												
0-5 cm	6.9	6.4	6.8	6.3	6.4	5.8	5.3	4.5	6.7	6.2	0.04	0.04
5-10 cm	6.9	6.3	6.9	6.3	6.4	5.7	5.1	4.2	6.7	6.1	0.06	0.05
10-15 cm	7.1	6.2	7.1	6.4	6.5	5.9	5.4	4.6	6.9	6.2	0.04	0.07
LSD $P < 0.05$	0.07	0.01	0.04	0.08	0.11	0.01	0.1	0.02	0.6	0.6		

[a] soil:water or soil:$CaCl_2$ solution (0.01 M) = 1:2.5 ratio.

[b] Mean and all the LSD values for Reps I, II, and III.

Table 24. Effect of tillage and residue management on soil organic C

Treatments	Rep I	Rep II	Rep III	Rep IV	Mean[a]	LSD[a] $P < 0.05$
	% Organic C					
No Till/Bare	1.29	1.03	1.12	1.07	1.15	0.06
No Till/Normal	1.57	1.42	1.36	1.16	1.45	0.05
No Till/ 2X Mulch	1.91	2.07	1.72	1.45	1.90	0.03
Chisel/Normal	1.60	1.37	1.42	1.13	1.46	0.03
Chisel/ Mulch	1.45	1.41	1.48	1.11	1.45	0.07
Moldboard Plow / Normal	1.21	1.09	1.07	1.02	1.12	0.04
Moldboard Plow / Mulch	1.49	1.17	1.44	1.49	1.37	0.07
LSD $P < 0.05$	0.03	0.03	0.04	0.03	0.22	
Depth of No Till / 2x Mulch						
0-5 cm	2.68	3.01	2.98	2.74	2.89	0.07
5-10 cm	1.91	1.29	1.03	1.06	1.41	0.02
10-15 cm	1.43	1.00	0.95	1.07	1.13	0.02
LSD $P < 0.05$	0.09	0.09	0.08	0.05	0.64	

[a] Mean and LSD values for Reps I, II, and III.

Statistical Analysis

The statistical analyses of the data obtained were performed by using Statistical Analysis System (SAS) computer language (Barr et. al., 1976). Replicate IV was the furthest from the gravel road and, as a result, lower values were obtained for pH, organic C, and the arylamidase activity. The results obtained from this replicate are only shown on Tables 23-25, but they were not included in any mean or statistical analysis to better reveal the effect of tillage and residue management on the soil parameters studied.

RESULTS AND DISCUSSION

To aid the discussion about the effects of tillage and residue management on the activity of arylamidase, the next two paragraphs will review the results reported by Deng (1994), related to the values of soil pH and organic C of these samples.

Table 23 shows that the soil pH in the replicates I, II, and III varied slightly due to the tillage and residue management treatments. For example, the pH values obtained from all the treatments ranged from 6.7 to 7.2 in water and 6.1 to 6.5 in 0.01 *M* $CaCl_2$ for replicate I. More variation was found between replicates than within treatments due to the location of the plots from the gravel road (see Materials and Methods). The values of soil pH were similar in different depth increments within each replicated plot of the no till / 2X mulch (NT2M) treatment.

The organic C content at the soil surface (0 to 5 cm depth) was significantly affected by the different tillage and residue management treatments tested. The greatest soil C content was obtained with the no-till / 2X mulch (NT2M), and the least with the no-till / bare (NTB) and moldboard / normal (MPN) treatments. The organic C content at the 0 to 5 cm depth was significantly greater than those of the subsurface soil samples (5-10 and 10-15 cm).

Results in Table 25 show that the activity of arylamidase was

Table 25. Effect of tillage and residue management on arylamidase activity in soils

Treatments	Rep I	Rep II	Rep III	Rep IV	Mean[a]	LSD[a] $P < 0.05$
	mg β-napthylamine kg^{-1} soil h^{-1}					
No Till/Bare	35	29	25	9	30	8
No Till/Normal	47	46	30	8	41	14
No Till/ 2X Mulch	42	53	40	8	45	9
Chisel/Normal	44	48	37	7	43	11
Chisel/ Mulch	46	46	56	17	49	14
Moldboard Plow / Normal	27	35	31	10	31	11
Moldboard Plow / Mulch	41	44	56	23	47	16
LSD $P < 0.05$	6	10	11	2	12	
Depth of No Till / 2x Mulch						
0-5 cm	80	82	56	25	73	18
5-10 cm	40	44	24	12	36	6
10-15 cm	29	28	22	16	26	5
LSD $P < 0.05$	8	4	17	3	21	

[a] Mean and LSD values for Reps I, II, and III.

significantly affected by the different tillage and residue management treatments tested. The values of arylamidase activity showed the following trend: CPM > MPM > NT2M > CPN > NTN > MPN > NTB. The treatments CPM, MPM and NT2M resulted in greatest arylamidase activity, with no significant differences among them (Figure 35). Thus, it seems that despite the tillage systems used, when it is complemented with mulch, mulching influences arylamidase activity. It is well known that enzymes are incorporated into the soil with the crop residue and that higher activities are observed due to the additional readily available substrates from the crop residues. In addition, the application of crop residues to the soil leads to better soil aggregation, and thus, higher water infiltration and soil water-holding capacity, and to the reduction of water evaporation, which influence the stability and production of enzymes in soils. The arylamidase activity values obtained with the treatments NTN and CPN were lower, but not statistically different, than those of the treatments CPM, MPM, and NT2M, because these treatments did not involve addition of crop residues (mulch) but the residues from the previous crop were not removed. In general, the treatment CPM increased the activity of arylamidase by 1.6-fold compared with the NTB or MPN treatments. The arylamidase activity values in the CPM treatment were 49 mg β-napthylamine kg^{-1} soil h^{-1} compared with 31 and 30 mg β-napthylamine kg^{-1} soil h^{-1} for the MPN and NTB, respectively. The treatments MPN and NTB showed the lowest arylamidase activity values, and these values were significantly different from the other treatments tested.

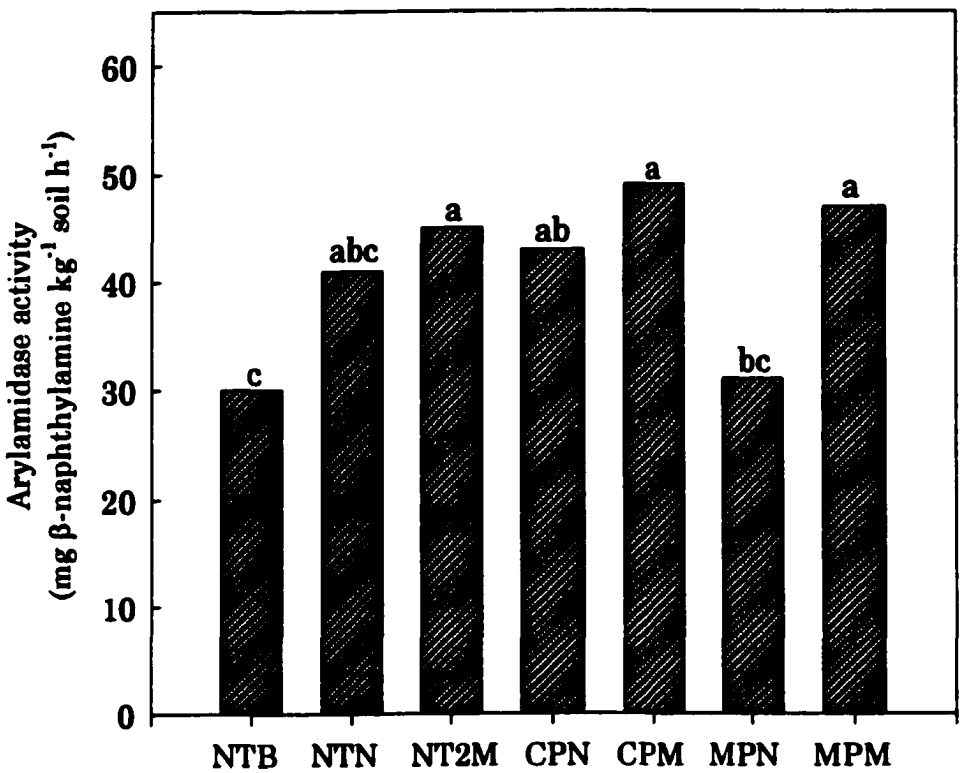


Figure 35. Effect of tillage and residue management on the activity of arylamidase (average of three replicated plots) in soils. Different letters indicate significantly different means at $P < 0.05$ according to LSD test

The activity of arylamidase decreased significantly with soil depth (Table 25) and the same trend was observed with the content of organic C with depth (Table 24). Figure 36 shows that arylamidase acitivity was significantly correlated ($P < 0.05$) with the organic C content in the samples taken at the 15 cm depth from all the treatments. These results agree with findings of other researchers that have also reported the enzyme activities are correlated with the organic C content of soils (Speir, 1977; Dick, 1984). The activity of arylamidase was also significantly ($r = 0.80$, $P < 0.001$) correlated to the content of organic C (g kg^{-1} soil) in 26 different surface soil samples tested in Part II.

Results showed that arylamidase activity is affected by soil pH_{CaCl2} ($r = 0.55$, $P < 0.05$), but not when the pH was measured with water (Figure 37). These results agree with those reported in Part V, where the activity of this enzyme was also significantly correlated ($r = 0.74$, $P < 0.001$) with the soil pH in one limed soil. Soil pH affects the microbial dynamic and community structure, and may influence enzyme induction in the microbial population. Also, soil pH affects the rate of the enzyme-mediated reactions by influencing the ionization of the substrate, cofactors, and the enzyme proteins.

Results of arylamidase activity were significantly affected by tillage and residue management practices which agree with those reported by others for other soil enzymes (Dick, 1984; Deng and Tabatabai, 1996 a,b, 1997). Thus, the results may suggest this enzyme is also a potential tool for soil quality research focused on monitoring the changes influenced by soil management practices.

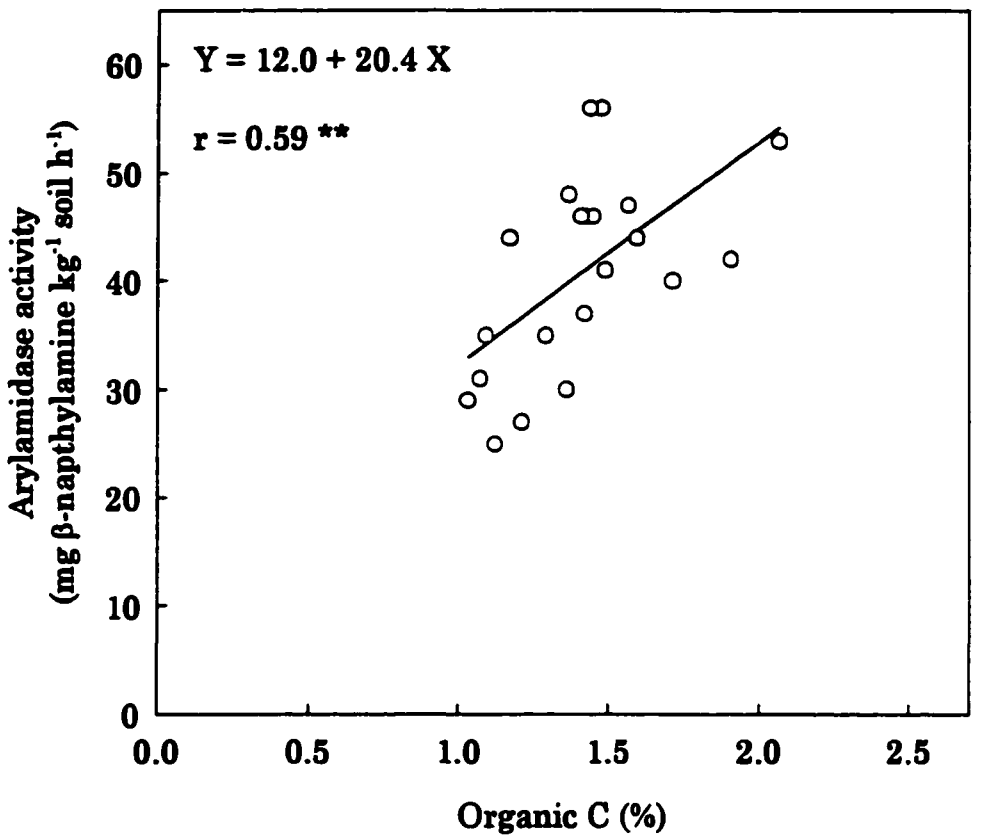


Figure 36. Relationship between arylamidase activity and organic C in soils

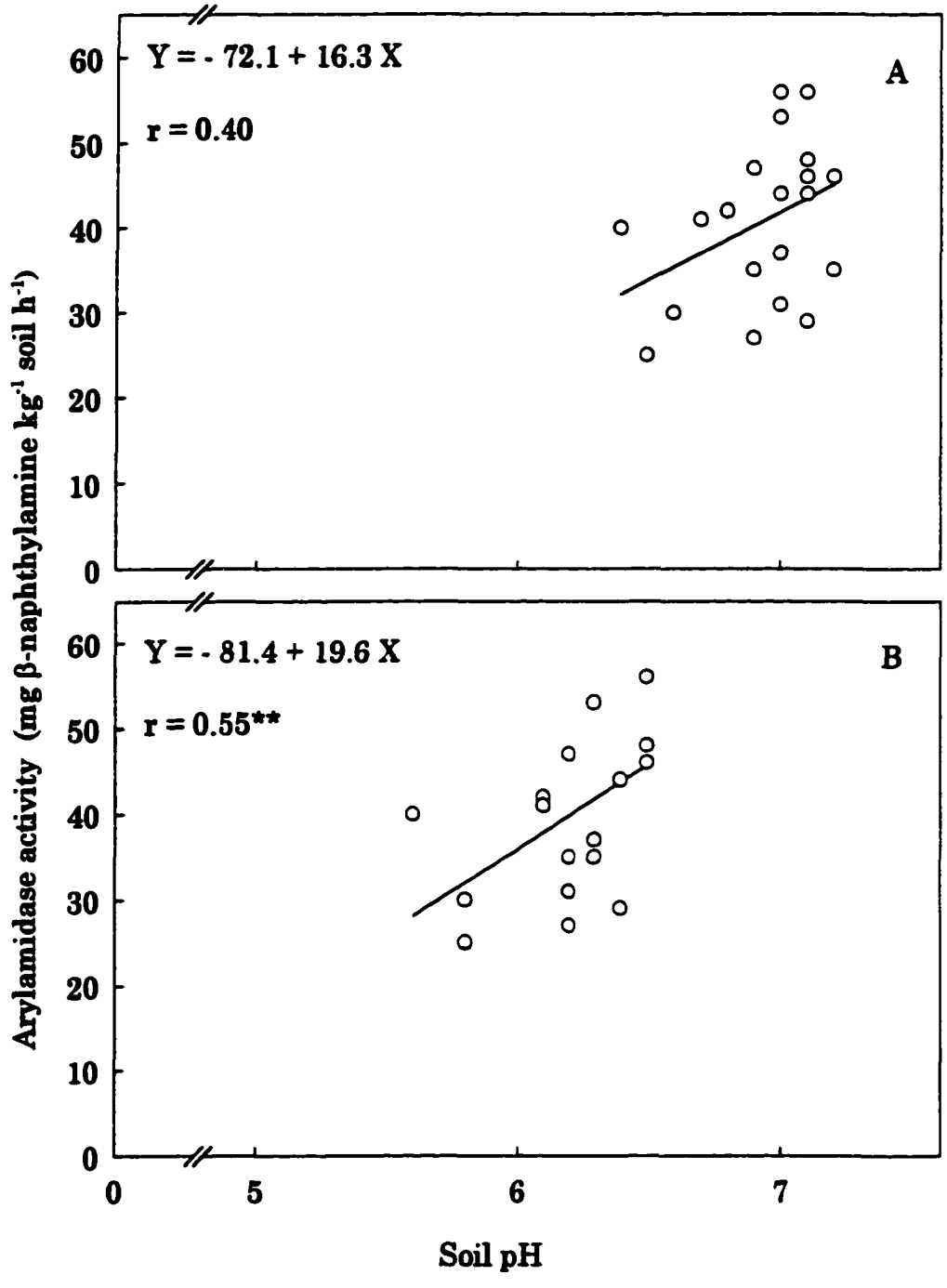


Figure 37. Relationships between arylamidase activity and soil pH determined (A) in water; and (B) in $CaCl_2$

SUMMARY AND CONCLUSIONS

The objectives of this study were: (1) to develop a method to assay the activity of arylamidase in soils, (2) to assess the factors affecting arylamidase activity in soils and its relationship to the amidohydrolases, (3) to assess the effect of trace elements on the activity of this enzyme in soils, (4) to determine the effect of toluene on the activity of arylamidase, (5) to study the substrate specificity of arylamidase, (6) to evaluate the effect of liming on the activities of 14 different enzymes including arylamidase, and (7) to evaluate the influence of tillage and residue management on the activity of arylamidase.

The findings can be summarized as follows:

1. The method developed to assay the activity of arylamidase in soils involves colorimetric determination of the β-naphthylamine produced when soil is incubated with L-leucine β-naphthylamide in 0.1 *M* THAM [tris(hydroxymethyl)aminomethane] buffer (pH 8.0) at 37°C for 1 h. The β-naphthylamine was extracted with ethanol and converted into an azo compound by reaction with *p*-dimethylaminocinnamaldehyde, and the absorbance of the color was measured at 540 nm. The method allowed for quantitative determination of the β-naphthylamine produced. This enzyme has its optimal activity at pH 8.0 and is inactivated at temperatures above 60°C. Preheating soil samples for 2 h at temperatures ranging from 20 to 120°C before assay showed that this enzyme is stable up to 40°C in field-moist soils and up to 60°C

in air-dried samples. The K_m values of arylamidase activity in seven surface soils ranged from 0.19 to 0.35 m*M*. The temperature response followed Arrhenius equation over the range from 20 to 50°C. The E_a values ranged from 30.6 to 49.8 kJ mol^{-1} for field-moist soils and from 26.2 to 32.4 kJ mol^{-1} for their air-dried counterparts. The ΔH_a values for air-dried soils ranged from 23.7 to 30.1 kJ mol^{-1}. The means of Q_{10} ranged from 1.32 to 1.71 (avg. = 1.44). The compounds toluene, formaldehyde, dimethylsulfoxide, $HgCl_2$, or iodoacetic acid inhibited, and autoclaving completely destroyed, the activity of this enzyme in soils. Arylamidase activity in soils ranged from 18 to 140 mg β-naphthylamine kg^{-1} of soil h^{-1} with coefficients of variations that were generally ≤ 4 % in all soils.

2. The activity of arylamidase was significantly correlated with the contents of organic C ($r = 0.80$; $P < 0.001$) and total N ($r = 0.71$; $P < 0.001$), and with clay ($r = 0.49$; $P < 0.05$), but not with the content of sand or the pH of the 26 surface soils examined. The activity of this enzyme was also significantly correlated with the activities of L-asparaginase ($r = 0.91$; $P < 0.001$), L-aspartase ($r = 0.90$; $P < 0.001$), urease ($r = 0.87$; $P < 0.001$), L-glutaminase ($r = 0.84$; $P < 0.001$) and with amidase ($r = 0.39$; $P < 0.01$) in the soils.

3. Among the 25 trace elements tested, Ag(I), Hg(II) and Cd(II) were the most effective inhibitors (55- 90%) of arylamidase activity in both air-dried and field-moist soils. The trace elements Co (II), Mg (II), Mn (II), B (III), and As (V) activated the activity of arylamidase in both air-dried and field-moist soils

while the metals W (VI) and Mo (VI) activated this enzyme in the air-dried soils, but acted as inhibitors in the field-moist soils.

4. Studies showed that toluene inhibits the arylamidase activity in soils. The K_m values for arylamidase in four soils using the Lineweaver-Burk plot ranged from 0.28 to 0.77 and 0.19 to 0.35 m*M* in presence and absence of toluene, respectively. The V_{max} values of this enzyme ranged from 16 to 81 and 22 to 100 mg β-naphthylamine kg^{-1} of soil h^{-1} in presence and absence of toluene, respectively. The E_a values of the reaction catalyzed by arylamidase in the soils in presence and absence of toluene ranged from 19.3 to 27.2 kJ mol^{-1} and from 26.2 to 32.4 kJ mol^{-1}, respectively. The ΔH_a values for arylamidase activity in four air-dried soils ranged from 16.3 to 24.1 kJ mol^{-1} and from 23.7 to 30.1 kJ mol^{-1} in presence and absence of toluene, respectively. The means of Q_{10} values for arylamidase in four soils for temperatures between 20 and 40°C ranged from 1.33 to 1.47 and 1.32 to 1.58 in presence and absence of toluene, respectively. Because both constants (K_m and V_{max}) of arylamidase were affected by the presence of toluene, the presence of such compound has a mixed type inhibition on arylamidase which indicates that toluene inhibits both the enzyme and the enzyme-substrate complex.

5. Studies on the substrate specificity of arylamidase showed that the amino acid moieties linked to β-naphthylamine affected the rate of hydrolysis of the substrate by this enzyme. The activity of arylamidase decreased as follows: alanine > leucine > serine > lysine> arginine = glycine = histidine > proline (not

hydrolyzed). The variation in the K_m and V_{max} values of arylamidase in soils for the different amino acid β-naphthylamides tested indicated that the amino acid moiety of the substrate affected the affinity of the enzyme to its substrate. The E_a values varied among the soils and were affected by the amino acid linked to the substrate, but in general, the values were within the ranges reported for other soil enzymes. The means of Q_{10} values for arylamidase activity in four soils for temperatures between 20 and 40°C ranged from 1.04 to 1.66 for all the amino acid moieties studied.

6. Studies revealed that the activities of the 14 different enzymes, assayed at their optimal pH of buffer, were significantly affected by lime treatments that ranged from 0 to 17920 kg effective calcium carbonate equivalent ha^{-1}. The enzymes α and β-glucosidases, α and β-galactosidases, amidase, arylamidase, urease, L-glutaminase, L-asparaginase, L-aspartase, alkaline phosphatase, phosphodiesterase, and arylsulfatase were significantly and positively correlated with soil pH, and acid phosphatase was significantly but negatively correlated with soil pH. The contents of C and N on surface samples were not significantly affected by the treatments but the soil pH was increased from 4.9 to 6.9.

7. The activity of arylamidase was significantly affected by tillage and crop residue placements. The greatest arylamidase activity was found with chisel mulch (CPM), moldboard plow mulch (MPM) and no-till double mulch

(NT2M), and the lowest with moldboard plow normal (MPN) and no-till bare (NTB).

8. The results from this work indicated that arylamidase is a potential indicator for health and soil quality, and in research dealing with the changes influenced by lime applications and by tillage and residue management. The results showed the potential of the enzyme to provide information that can aid in selecting better strategies for the manipulation of such management practices for improving soil quality, and the fertility and productivity of soils.

APPENDIX

Table 26. Activitities of the enzymes involved in the N cycle in 27 Iowa surface soils used in part II[a]

Soil	Aspartase	Amidase	Urease	L-asparaginase	L-glutaminase	Arylamidase
	mg NH_4^+-N kg^{-1} soil 24 h^{-1}		mg NH_4^+-N kg^{-1} soil 2 h^{-1}			mg β-naphthylamine kg^{-1} soil h^{-1}
Storden	62.8	74.7	23.6	8.6	97.8	7.5
Ida	44.2	276	43.4	11.5	49.1	46.9
Hayden	57.7	134	55.0	9.0	55.5	12.9
Hagener	84.7	256	50.6	15.1	145	17.3
Weller	87.3	339	28.0	13.9	91.9	26.2
Luther	56.5	184	24.6	9.6	114	16.6
Gosport	176	615	122	33.8	369	54.5
Downs	149	474	91.2	26.5	223	25.6
Fayette	104	339	58.9	27.7	258	32.5
Pershing	103	386	29.4	20.5	168	22.7
Clinton	189	864	102	47.9	369	51.5
Edina	143	380	62.7	21.1	288	31.9
Tama	125	227	79.1	40.9	358	42.6
Marshall	107	429	81.0	16.4	139	23.6
Shelby	230	653	137	36.5	503	39.5
Monona	236	567	163	54.2	496	75.7
Sharpsburg	168	608	131	31.8	383	37.5
Muscatine	99	299	74.8	24.1	260	32.5
Grundy	160	144	61.3	27.3	309	36.0
Ames	262	677	85.4	67.5	678	44.7
Clarion	350	657	70.9	54.2	413	53.6
Webster	373	648	122	50.1	444	75.6
Nicollet	313	301	205	69.0	668	87.6
Canisteo	478	389	385	87.9	722	115.7
Okoboji	273	636	147	47.9	448	72.6
Harps	517	386	204	101	711	107.7

[a] Except for the values of actvitiy of arylamidase, the rest of the data is from Senwo Ph.D. thesis.

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